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We have modified a piston impact model of brain injury in the anesthetized rat. The focal right sensorimotor cortical injury produced a consistent contralateral hemiparesis plus long term memory and spatial localization deficits. A 4 test battery indicated that sensorimotor function spontaneously improved by 35 days largely by behavioral adaptation. Sensorimotor recovery was biphasic; possibly different mechanisms underlie early and late behavioral improvement. Long term memory deficits recovered by 17 days. Spatial localization deficits caused rats to exhibit stereotypic strategies in maze tests. Histological studies showed widespread cell injury far from the focal lesion evident up to 8 weeks after the brain injury. Transsynaptic axonal degeneration occurred throughout the entire subcortical motor system involving even the cerebellum. Free fatty acids and diacylglycerol increased and brain phosphorous decreased not only around the focal cortical injury but widely throughout the brain. These chemical changes were persistent indicating that neural degeneration was occurring for at least 35 days after injury.

Severe cortical impact sufficient to cause apnea was associated with severe disruption of the floor of the fourth ventricle adjacent to medullar respiratory nuclei. Mechanical disruption of neural respiratory circuits may account for irreversible apnea.

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FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army. Much of the work presented is "in progress". Conclusions drawn from "in progress" work are our best understanding of the various biological processes based on current data. As more data becomes available in the future our conclusions may change.

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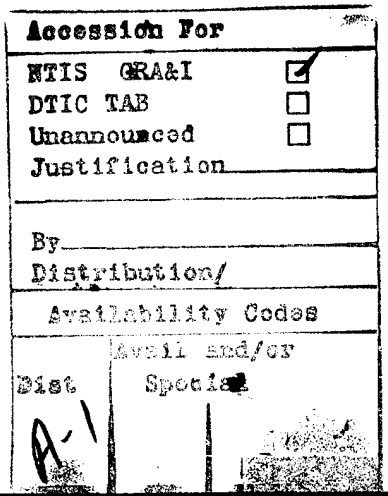

Michael E Carey MD 31 May, 1995
PI- Signature Date

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BACKGROUND

Nature of the Problem

In combat, head wounds account for almost half of all single wound deaths. Since World War II neurosurgical mortality has approximated 10%, indicating no overall improvement in the lethality of brain wounds incurred in combat [1]. In an attempt to alleviate this problem the US Army Medical Research and Development Command sponsored a project to develop an experimental model of brain wounding so that better treatments for brain wounded soldiers could be developed. This project was halted by animal zealots [2]. The present brain injury project being conducted at Louisiana State University Medical Center is an attempt to continue the U.S. Army's efforts to develop better treatments for brain injury. In this new effort we modeled brain injury in the anesthetized rat by impacting the right sensorimotor area with a pneumatically driven piston. Owing in no small measure to the animal activists, most brain trauma work around the world is now done in the anesthetized rat using fluid percussion, weight drop or a piston impact.

Previous Work on Brain Trauma in the Rat by Other Laboratories

Since the early 1980s brain trauma has been extensively evaluated in smaller animals, notably the rat. Brain injury has been produced by dropping a weight on the exposed dura [3] by fluid percussion [4-6] either in the midline or laterally or by a computer controlled pneumatic impactor [7,8]. Numerous investigators have taken up these models to study a wide variety of post traumatic effects and to evaluate means to attenuate the traumatic injury. Documenting behavioral changes after injury and treatment has been an important element in many of these studies. Weight drop, piston impact, and high levels of fluid percussion invariably produce an area of necrosis at the injury site. Histologic changes and blood-brain barrier breakdown associated with these injuries were evaluated by several investigators [3,9-12]. Sutton, using a pneumatic impactor as do we, found that cortical edema at 6 and 24 hours post injury and the amount of cortical necrosis at 1 week and 1 month after injury were a function of cortical compression depth. The blood-brain barrier has been shown to close around the injury site 72 hours after injury.

Lateral fluid percussion injury reduces blood flow widely throughout the brain for up to one hour after injury and produces longer lasting reductions about the impact site [13]. Brain energy metabolism is also impaired widely throughout the brain and energy impairments may last up to 9 days after injury. Immediately after injury cerebral glucose utilization increases but by 6 hours after injury most areas in the ipsilateral cortex demonstrate metabolic depression [14-17]. If cerebral blood flow

does return to normal within an hour or so after injury as Yamakami's data suggest but brain metabolic changes last for days, **brain injury produces a long lasting uncoupling of cerebral blood flow and metabolism.**

Impact injury causes increased brain prostaglandin synthesis, free radical formation, activation of brain phospholipase A_2, and increases in brain free fatty acids in and adjacent to the brain injury site [18-22].

Brain electrolytes also change following impact injury: brain extracellular K^+ and Ca^{++} increase and Mg^{++} falls. These data suggest that spreading depression (deplorization) accompanies traumatic brain injury. Entry of Ca^{++} into cells is part of the pathologic process leading to cell death [23].

Cortical impact is also accompanied by release of brain excitatory amino acids (glutamate, aspartate) which are thought to potentiate injury. N-methyl-D-aspartate (NMDA) receptors decrease after injury indicating that these receptors down-regulate in response to overstimulation by glutamate and possibly by other ligands. NMDA receptor blockers prevent or attenuate histological evidence of damage [24-26]. **Clearly, after impact injury excitatory amino acid changes throughout the brain are an important story line because they have been associated with enhancement of brain damage.**

Catecholamine changes occur about the injured brain area and in other brain areas as the hypothalamus. Such changes may last many days after impact injury. Following traumatic cortical injury alpha adrenergic receptors decrease in the cortex [27-29]. Some investigators have felt that the alpha noradrenergic system plays a pivotal role in both the pathology and treatment of motor deficits following sensorimotor cortical injury [30].

Acetylcholine turnover is increased in the dorsal pontine tegmentum and thalamus minutes to hours after fluid percussion injury while it is decreased in the amygdala. Possibly, post traumatic pontine activation contributes to post injury behavioral suppression [31].

Protein kinase C increases in the cortex and hippocampus after lateral percussion injury. This increase as well as post injury increases in glutamate may be blocked by the drug MK-801 [32].

A most provocative series of microstimulation experiments demonstrated that following cortical injury recovery of neural function does not take place at cortical levels either ipsilateral or contralateral to the lesion [33]. **The logical conclusion from these experiments is that neurologic improvement following cortical injury must occur at subcortical levels.**

Sensorimotor cortical lesions produce cerebellar biochemical changes as well. Cerebral cortical trauma reduces norepinephrine release bilaterally in the cerebellum for at least 48 hours while cerebellar norepinephrine infusions improve motor deficits after such injury [34,35].

While most traumatic brain injury studies have concentrated on post injury motor deficits, memory, spatial localization, and learning deficits have also been documented [36-40].

In addition to ascertaining basic mechanisms underlying brain injury, investigators have also sought to ameliorate traumatic brain injury through cerebral hypothermia [41] but more notably by drug therapy. Early on it was discovered that amphetamine or norepinephrine could improve motor performance after injury [42,43]. Scopolamine also improves function after traumatic brain injury suggesting that brain trauma is associated with excessive muscarinic cholinergic activation [44,46]. Various NMDA receptor blockers [47,48,49], calcium channel blockers [50,51], magnesium sulfate [52], 21 aminosteroids [53]; morphine [54], naloxone, and the TRH analog YM14673 [55] all improve some functional aspect following traumatic brain injury.

Previous Work on Brain Trauma by Our Laboratory

Our prior work on missile injury of the brain indicated that when 2.4 Joules of energy were deposited in the right cerebral hemisphere of the anesthetized cat, two thirds of such animals sustained a fatal apnea even though the missile track was 1-2cm away from the brain stem [56]. Clearly, brain stem dysfunction was occurring by some indirect means. If ventilatory support were given many apneic cats would resume breathing and recover neurologically so that they were indistinguishable from unwounded animals. We hypothesized that brain injury may be considered as involving two separate systems: 1) damage to the brainstem containing respiratory and cardiac control mechanisms; 2) damage to the cerebral cortex and hemispheral white matter. **Brain stem damage either direct or indirect determines living or dying; cortical/hemisphere damage determines neurologic residua.** We therefore divide our present work into two segments: 1) cortical damage without brain stem effects in which we do not evaluate the brain stem; 2) cortical damage causing brain stem dysfunction particualrly cardiorespiratory changes where we do not evaluate the cortex.

PURPOSE OF PRESENT WORK

The overall purpose of our experiments is to develop and establish a reproducible model of traumatic brain injury in the rat where biological changes can be correlated with behavioral recovery or the lack thereof. Biological and behavioral characterization of this traumatic brain injury will allow the testing of newer neuroprotective drugs or combinations of drugs

to ameliorate brain injury. Despite the immense amount of work already done on neuroprotective agents, currently there is no drug on the market which has been shown to effectively ameliorate human brain injury.

METHODS

I. OVERVIEW OF APPROACH TO PRODUCE CORTICAL INJURY

Humans often have a contralateral hemiparesis after brain injury. To make our laboratory model more relevant to humans our experiments have centered around producing a left hemiparesis in the rat by damaging the entire right sensorimotor cortex, figure 1.

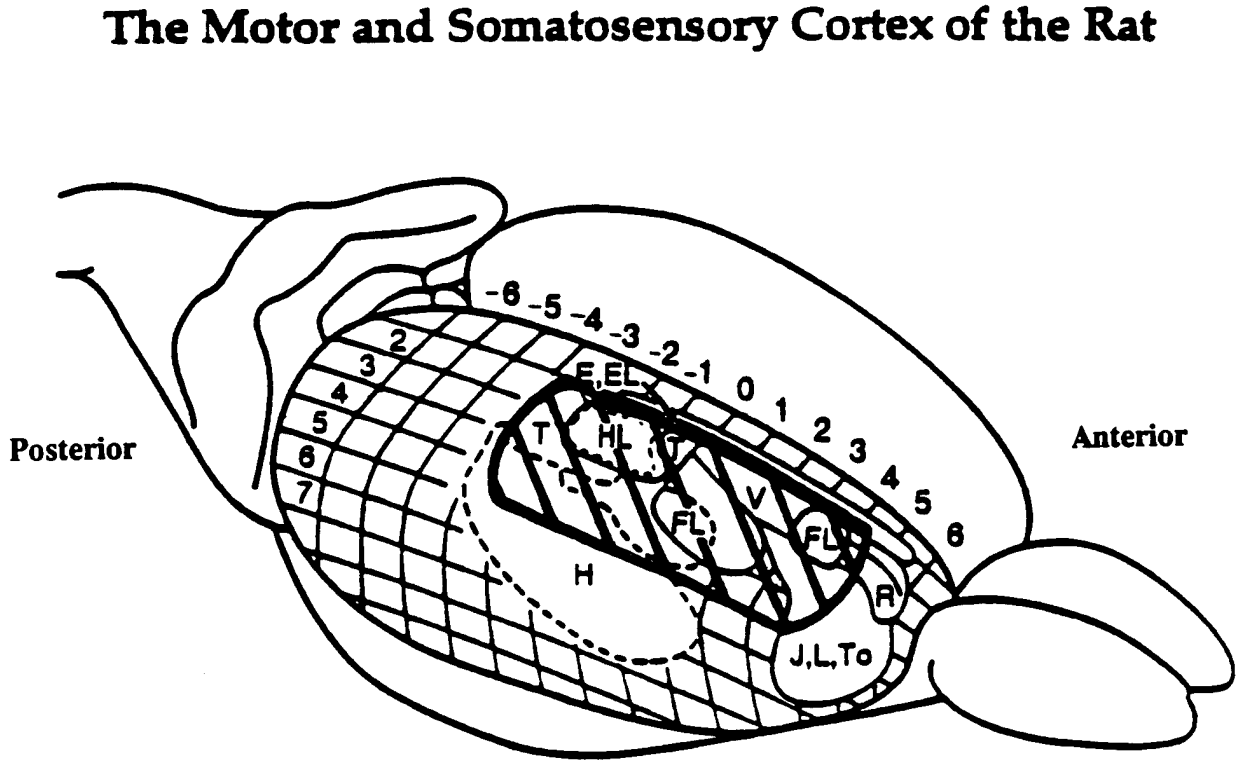


Figure 1 : Areas of rat brain injured by our 4mm X 8mm piston impact tip. Motor areas (solid lines) and primary somatosensory areas (broken lines). FL=forelimb, HL=hindlimb, E=eye, EL=eyelid, V=vibrissae, T=trunk, R=rhinarium, J=jaw, L=lip, To=tongue, H=head.

Modified from [57] with permission.

Brain damage is created in the anesthetized rat by a piston striking the exposed right sensorimotor dura. In developing the model we gave great consideration to the extent of cortical injury and the type of anesthetic to be used. Details of these considerations including physiological results following isoflurane or urethane anesthesia can be found in our quarterly report of 2 February, 1994. We evaluated different depths of dural depression by the piston and determined that isoflurane anesthesia, an impact speed of 5.2 m/sec, and dural depression of 1 mm were optimal to produce right cortical injury resulting in a sustained left hemiparesis. Dr. Soblosky has used a 4-test battery to measure motor recovery after this injury and 2 radial arm maze tests to evaluate post-injury memory dysfunction.

Dr. Soblosky provides brain injured rats to Dr. Bazan's laboratory for brain lipid determinations and to Dr. Matthews for histological studies.

We feel that a multidisciplined approach to the problem of traumatic brain injury is the most efficient, scientifically speaking, because each study group interacts and contributes ideas to the others. The whole is greater than the sum of its parts.

II. SPECIFIC METHOD OF PRODUCING RIGHT SENSORIMOTOR CORTICAL INJURY

An overnight fasted rat was anesthetized with isoflurane (1.5%) with $N_2O:O_2$ /5:1). The rat was then placed in a stereotaxic instrument. Under sterile conditions we made a midline scalp incision and right sided craniectomy 5 mm wide by 9 mm long. The craniectomy began 1 mm lateral to the midline and extended 5 mms further laterally. It was placed 4.5 mm anterior and 4.5 mm posterior to the coronal suture (i,e. the craniectomy was 9 mms in length).

We then moved the rat while still in the stereotaxic frame under a computer-interfaced injury device fitted with a 4 mm x 8 mm pneumatic piston impact tip which impacted the exposed dura at 5.2 m/sec, depressed it 1 mm, and contused the underlying brain.

During the experiments all rats spontaneously breathed. Body and brain temperatures were maintained within normal limits by a homeothermic heating blanket and a heat lamp. After injury bleeding was stopped, the scalp sutured, and the rat placed in a warmed oxygenated chamber until fully awake. The animal was then returned to a holding cage and frequently observed up to four hours after injury. No fully awake rat ever appeared in distress. When the rat was eating and drinking, it was returned to its home cage.

Uninjured, control rats were treated exactly the same way except for a slight modification in the craniectomy procedure.

The usual 5 mm X 9 mm craniectomy incorporates the coronal suture which adheres to the underlying dura. Even with utmost care dissecting the coronal suture periosteum from the dura results in minor motor deficits clearly evident on our motor behavior tests. Such minor motor deficits are greatly overshadowed by the major motor deficits caused by piston impact so are unimportant in injured animals. These minor deficits, however, confound behavioral testing in control, uninjured animals. To obviate this effect our control animals had two craniectomies. One extended from about .5 mm to 4 mm posterior to the coronal suture and the other .5 mm to 4 mm anterior to it. We did not remove the connective tissue which attaches the dura to the coronal suture line. We have had great success with this method and only a few control rats exhibited any subsequent motor deficits. Such rats have been excluded from our various studies.

Control and injured animals were euthanized at varying times after injury for histological, behavioral, or neurochemical studies. In all instances sacrifice was by intraperitoneal (i.p.) pentobarbital (50 mg/kg). After sacrifice brains were processed differently for either histological or biochemical studies.

A. <u>For histologic studies</u> all rat brains were fixed by trans cardiac perfusion of appropriate agents. When histological preparation was done at LSU we removed the brains and sent them to the Department of Anatomy for processing. Histological preparation of other brains was done commercially (Neuroscience Associates, Knoxville, TN). Brains of these rats were fixed by transcardiac perfusion with 4% paraformadehyde buffered with .067 M sodium cacodylate with 4% sucrose immediately after sacrifice. They were then decapitated and the labeled heads sent to the Neuroscience laboratory for processing.

B. For neurochemical studies in Dr. Bazan's laboratory rats were anesthetized by ether and sacrificed by focused microwave irradiation. Some rats were anesthetized with ether, decapitated and their heads immediately frozen in liquid nitrogen. In all cases brains were removed for chemical analyses by personnel in Dr. Bazan's laboratory.

C. Animals used in behavioral studies were ultimately sacrificed by i.p. pentobarbital (50 mg/kg). Their brains were fixed by transcardiac perfusion and subsequently removed and stored.

D. Rats in which brain amino acids and catecholamines were measured were anesthetized with i.p. pentobarbital (50 mg/kg) and decapitated. Their brains were removed within 1 minute, and placed in a -70° C freezer. Afterwards the brains were sliced and selected brain areas punched out and processed for HPLC analysis. Only preliminary work in this area has been done. No

brain biogenic amines/catecholamine data will be presented in this report.

RESULTS OF CORTICAL INJURY EXPERIMENTS

1. Physiological Studies

Impact causing a 1 mm cortical depression is accompanied by a brief, mild hypotensive response but neither heart nor respiratory rates are significantly affected, figure 2. The data are means with SEM, n=5. Blood gas and pH and blood glucose levels are also unaffected by this injury. This cortical injury produces no significant affect on the animal's ability to eat or appetite.

Figure 2a MEAN ARTERIAL BLOOD PRESSURE AFTER A 1mm CORTICAL DEPRESSION

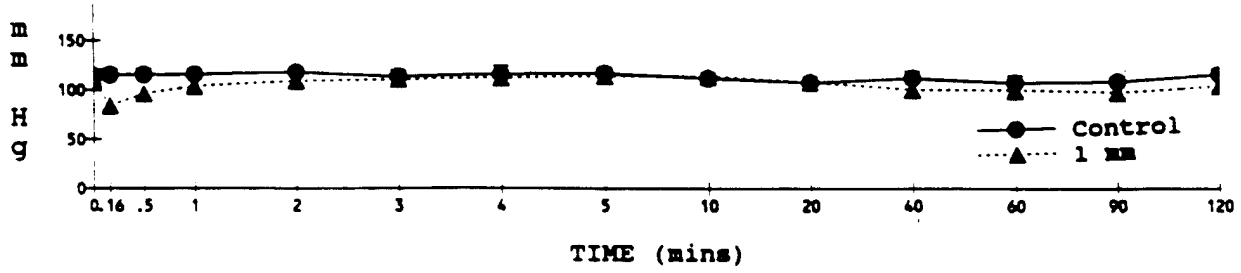


Figure 2b HEART RATE RESPONSE AFTER A 1mm CORTICAL DEPRESSION

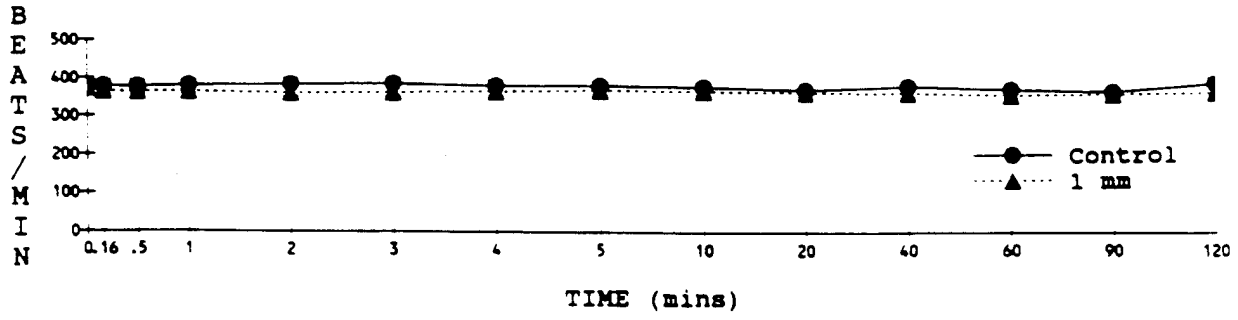
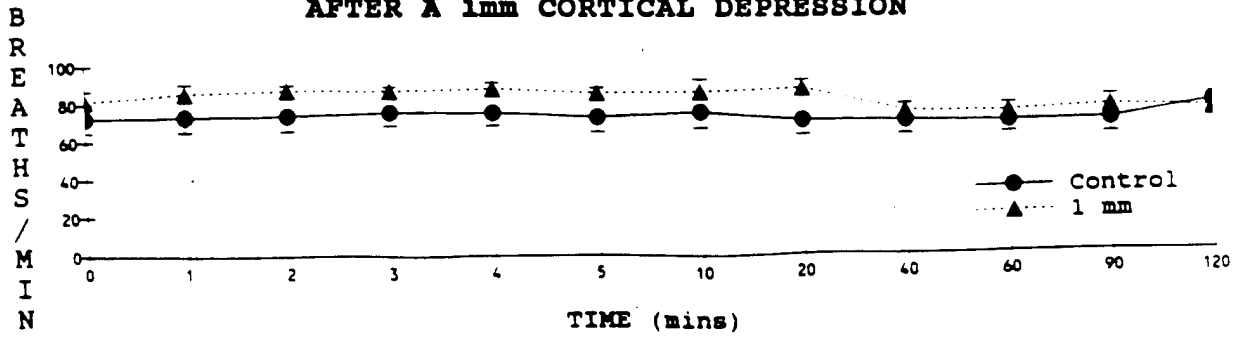


Figure 2c RESPIRATORY RATE AFTER A 1mm CORTICAL DEPRESSION



Discussion of physiologic studies

We feel it important to make a traumatic cortical lesion unconfounded by systemic cardiovascular or respiratory changes which could add the secondary insults of global ischemia or hypoxia to the traumatic cortical lesion itself. The 1 mm dural depression causes extensive cortical injury and produces significant neurobehavioral deficits. Owing to the absence of brain stem effects we can exclude the confounding effects of hypo or hypertension or hypoxia from apnea from contributing to this lesion. Furthermore, piston impact depressing the dura only 1 mm does not lacerate the dura. Brain herniation through a lacerated dura does not occur thus ensuring that local ischemia-hypoxia about the injury site does not contribute to the traumatic injury. The only uncontrollable variable which could affect the extent of injury from animal to animal would be an associated subdural or intracerebral blood clot. Large subdural or intraparenchymal blood clots do not usually occur following 1 mm dural depression.

2. Brain Sections and Histological Studies (Dr. Matthews)

A. Low Magnification Brain Sections

1) Mantle

Forty-eight hours after injury, the silver stains show that mantle at the impact site appears disrupted, figure 3, top. By 8 weeks the mantle has thinned considerably and the ipsilateral ventricle had undergone compensatory dilitation, figure 3 bottom. Figure 4 shows maximal, average, and minimal brain lesions observed in 11 injured rats sacrificed at 8 weeks.

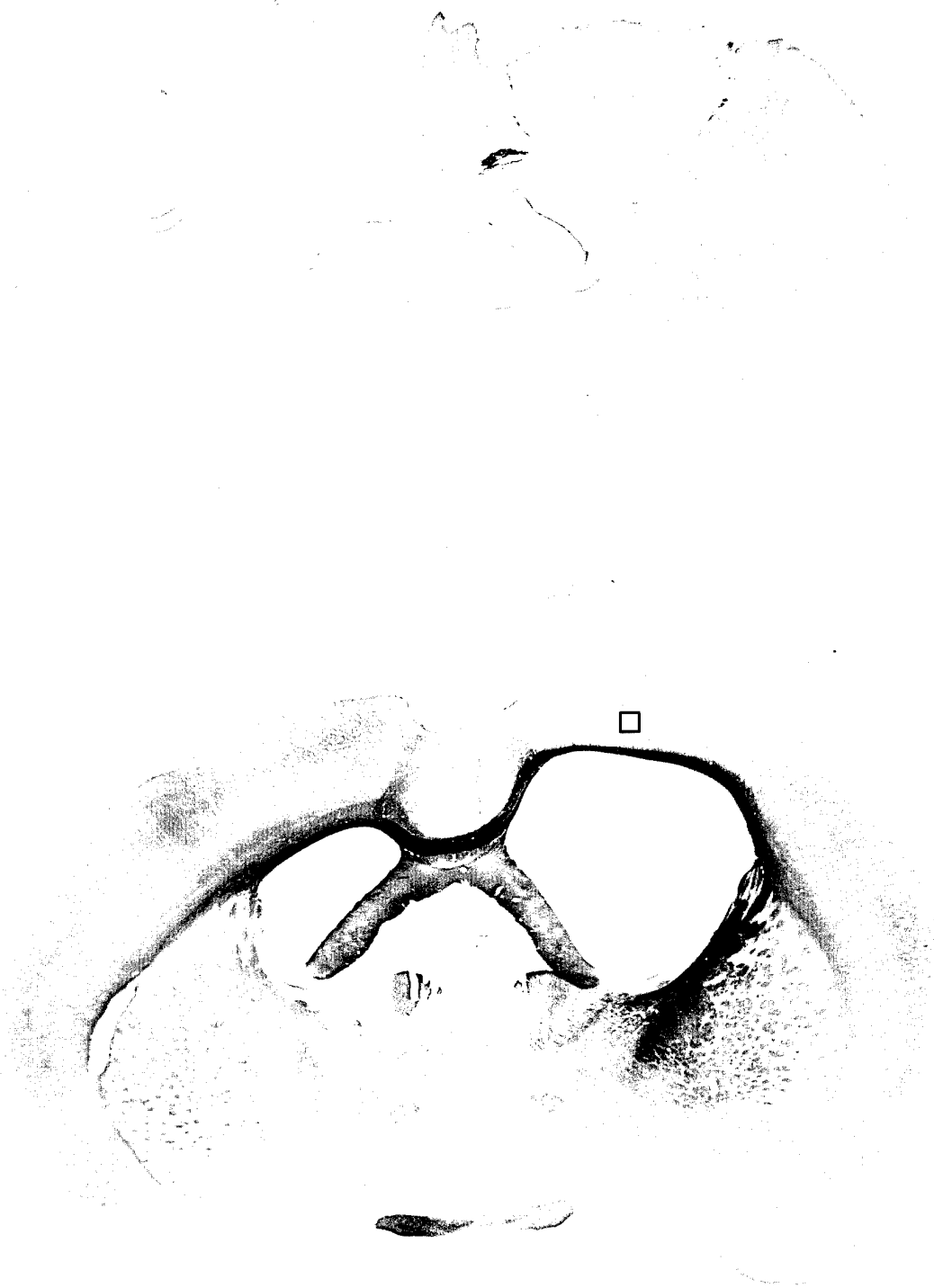


Figure 3. Comparison of cortical mantle thickness at 48 hrs. (top) and 8 weeks (bottom) after injury. Eight weeks after injury there is considerable thinning of the cortical mantle.

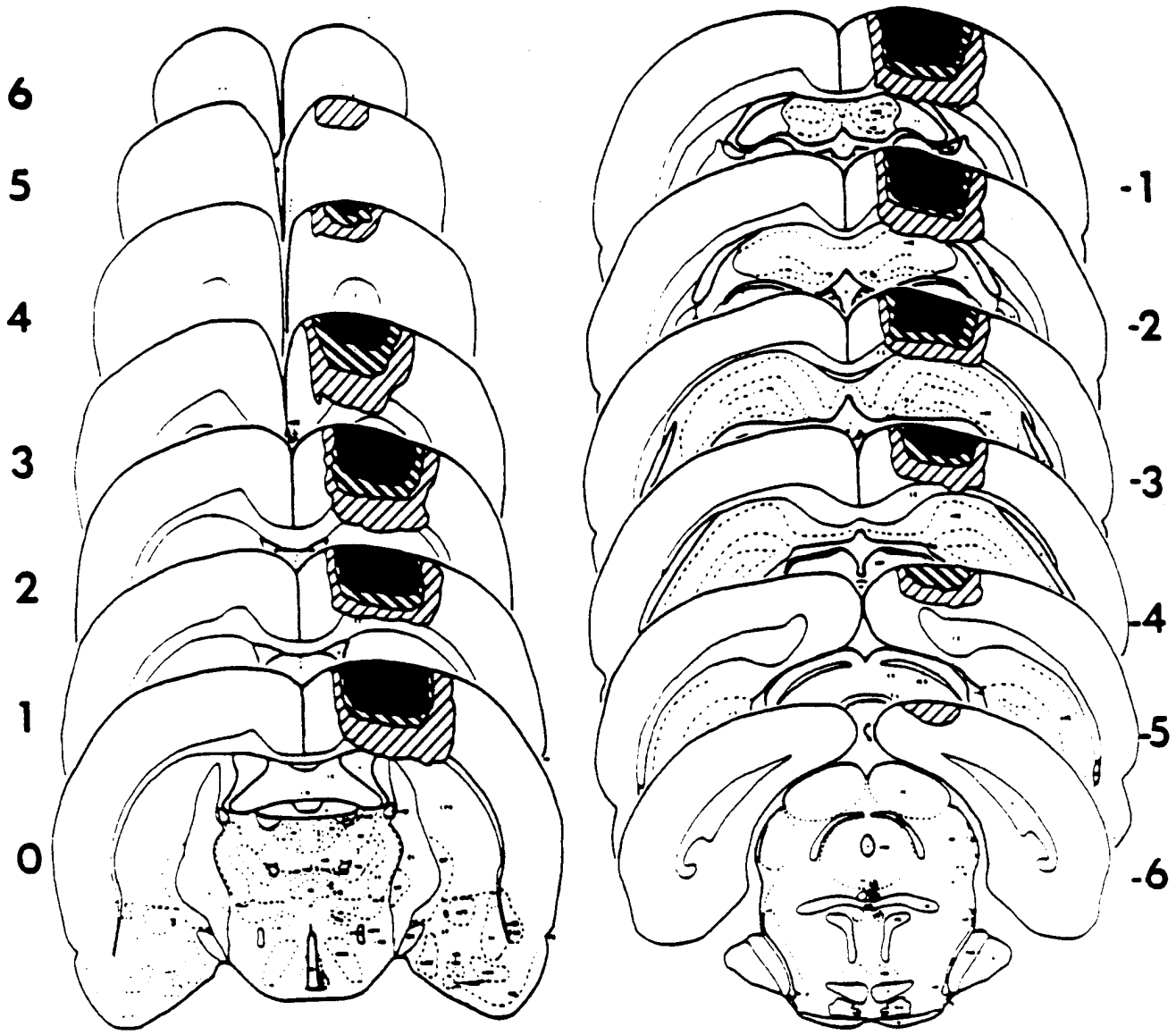


Figure 4. Drawings taken from the Atlas of the Rat Brain (Pelligrino, Pelligrino and Cushman). Selected coronal sections at one millimeter intervals through the brain from stereotaxic coordinates +6.0 to -6.0. The depth of damage was variable among rats (n=11). The maximum depth of injury is indicated by the light hatching. The most darkly shaded area indicates the minimal amount of cortex destroyed and the intermediate area indicated by the medium hatching illustrates the average amount destroyed by the impact injury.

Ventricles

Examination of ventricular size is most easily accomplished where internal brain structures constituting ventricular boundaries are not disturbed during specimen processing. For instance, the fornix and hippocampus are subject to tearing during processing. This makes estimation of or measuring ventricular areas impossible in some brain sections. Figure 5 shows the anterior lateral ventricles in the region of the striatum and anterior commissure. The top view shows a brain 48 hours after injury. Both ipsilateral and contralateral ventricles are small. Eight weeks later not only is the ventricle ipsilateral to the lesion dilated owing to mantle loss (as expected) but the contralateral ventricle is dilated as well.

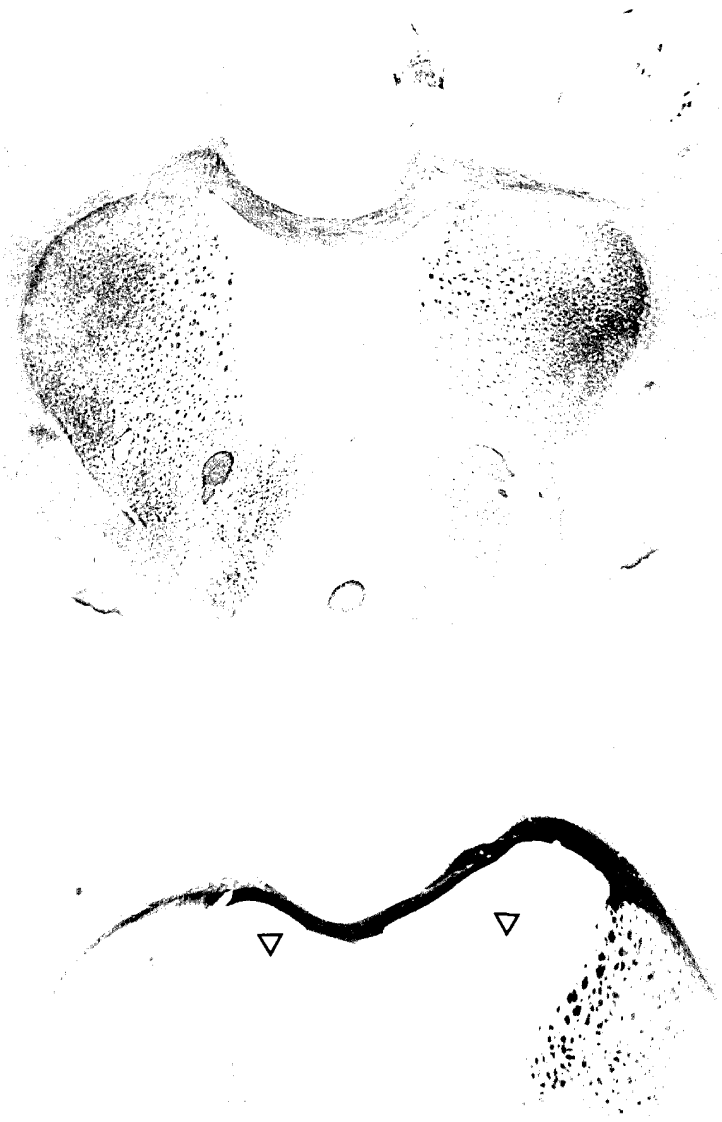


Figure 5. Comparison of anterior lateral ventricles at 48 hrs. (top) and 8 weeks (bottom) after injury. Eight weeks after injury both ventricles are enlarged (open triangles). The ipsilateral ventricle is more dilated because of the large amount of cortical mantle loss.

Discussion of Low Magnification Brain Sections

The area of cortical impact is reproducable from rat to rat. The amount of mantle thinning may vary somewhat but the pattern and density of axon degeneration appear identical in all rats with cortical injury (See Section C below). This indicates that any remaining cortex at the impact site is probably not functional. Contralateral ventricular enlargement suggests wide spread cell loss throughout the brain consequent to the focal traumatic impact. It could also represent a mild, post traumatic communicating hydrocephalus.

B. Quantitative Cell Counts

Normally neurons and glia stain lightly with cresyl violet-luxol fast blue as prepared in the histology laboratory, LSU Department of Anatomy. After injury, however, many cells about the impact site and throughout the brain stain darkly with this stain, figure 6. Electron micrographs show that shrunken neurons have severely deranged internal structure, figures 7A, 7B.

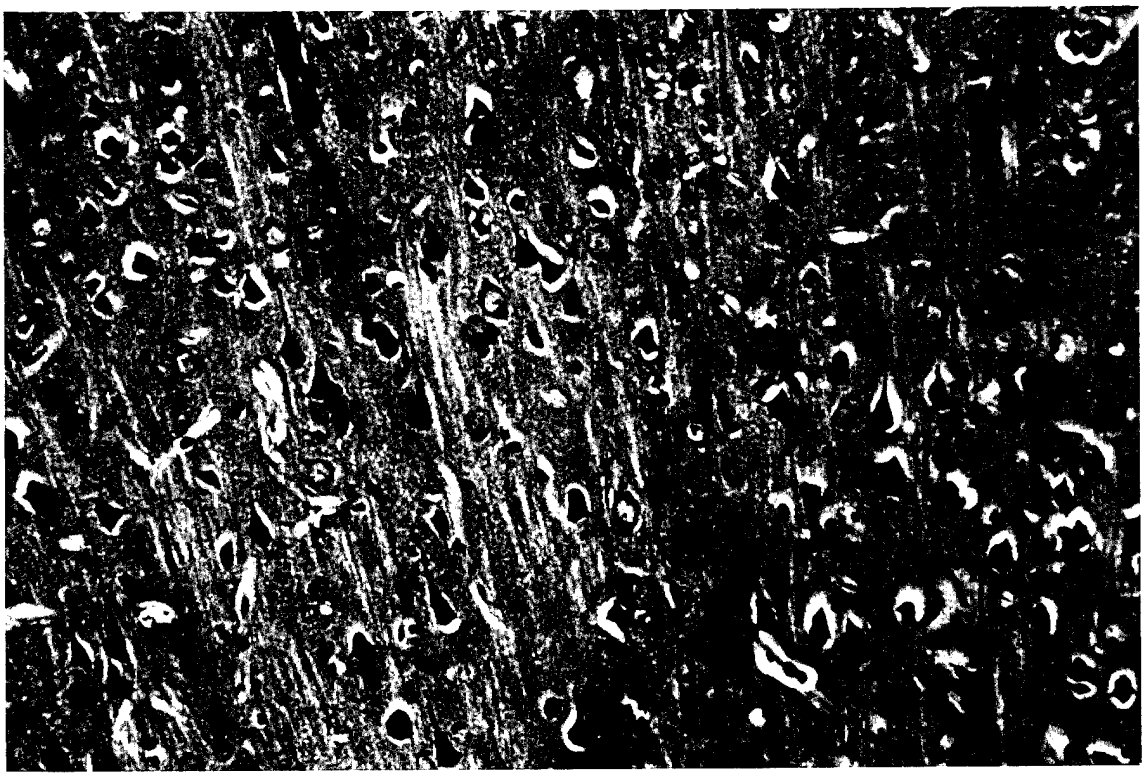


Figure 6: Brain section (10x). Unaffected neurons have distinct pale nuclei surrounded by a bluish-pink cytoplasm . Affected neurons have a dark bluish-green cast and their nucleus, if apparent at all, is indistinct .

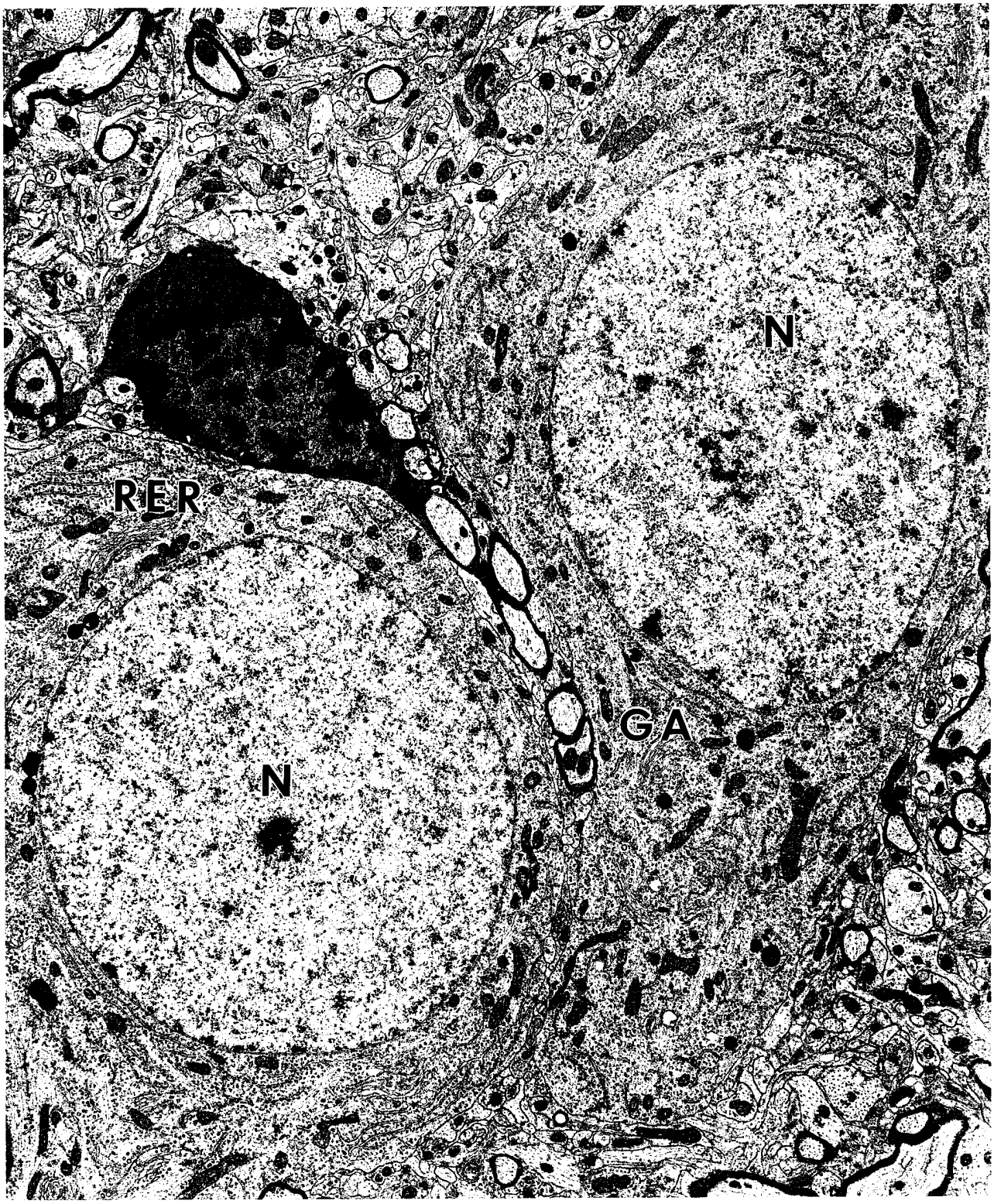


Figure 7A: Two normal neurons and an adjacent oligodendrocyte are shown in the normal cortex. They have an electron lucent nucleus (N) delineated by a distinct nuclear membrane and surrounded by a moderately electron dense cytoplasm filled with rough endoplasmic reticulum (RER), Golgi apparatus (GA), and scattered mitochondria.

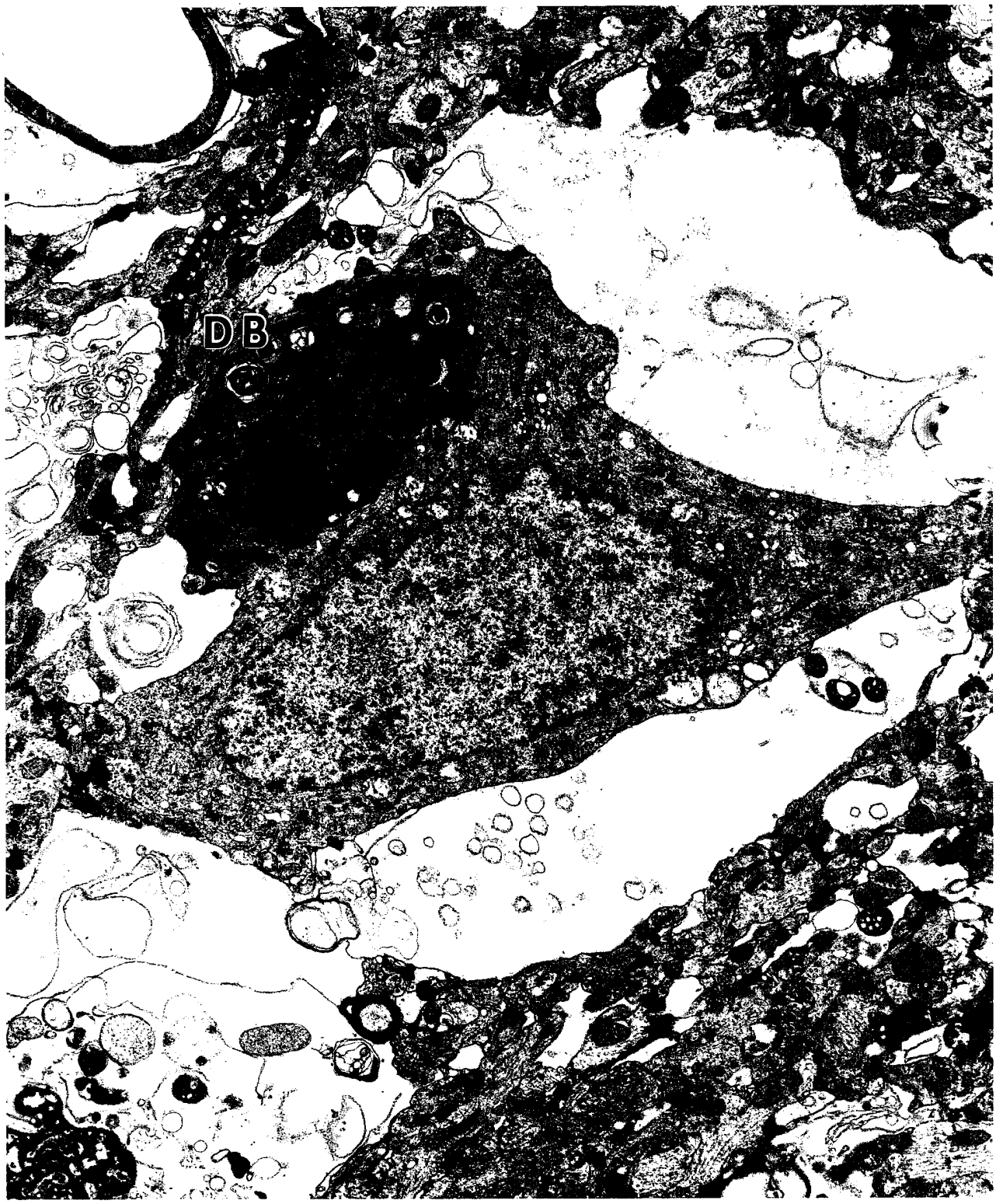


Figure 7B: Ipsilateral cortex. Eight week survival. A degenerating neuron with adjacent reactive microglial cell characterized by several dense bodies (DB). The neuron is moderately electron dense and displays several ruptured mitochondria. Extensive retraction of neuropil has taken place and degenerating synaptic elements can be observed. This image supports the light microscopic data which suggests that neuronal darkening is a continuous process that propagates over a long period of time and is accompanied by further synaptic degeneration.

We counted injured (darkened) neurons widely throughout the brain in 3 areas bilaterally, figure 8.

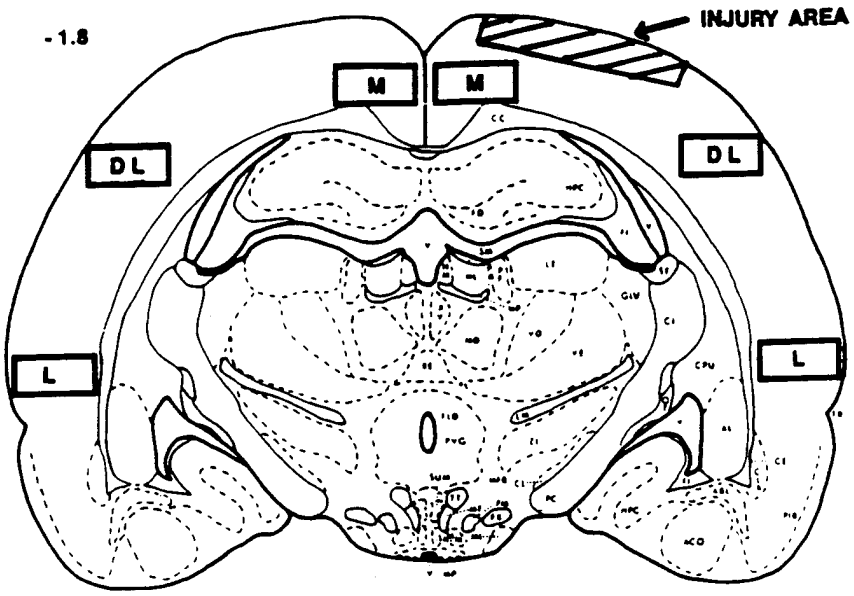


Figure 8: Two hundred and fifty to 350 cells were counted in each of 3 brain areas from both cerebral hemispheres after brain injury. M= medial; DL= dorsolateral; L= lateral

Sampling extended from 5.4 mm anterior to the coronal suture to 5.0 mm behind this point. Between 250 and 350 cells were counted for each determination. Cell counts were done on rats sacrificed 4 hours, 1 week, 4 weeks, and 8 weeks after injury. Three or 4 rats were counted for each time point. Figures 9A, 9B, 9C and 9D show the percent of damaged cells throughout the brain following impact injury. Uninjured brains showed maximally 1 to 2 percent darkened (injured) cells.

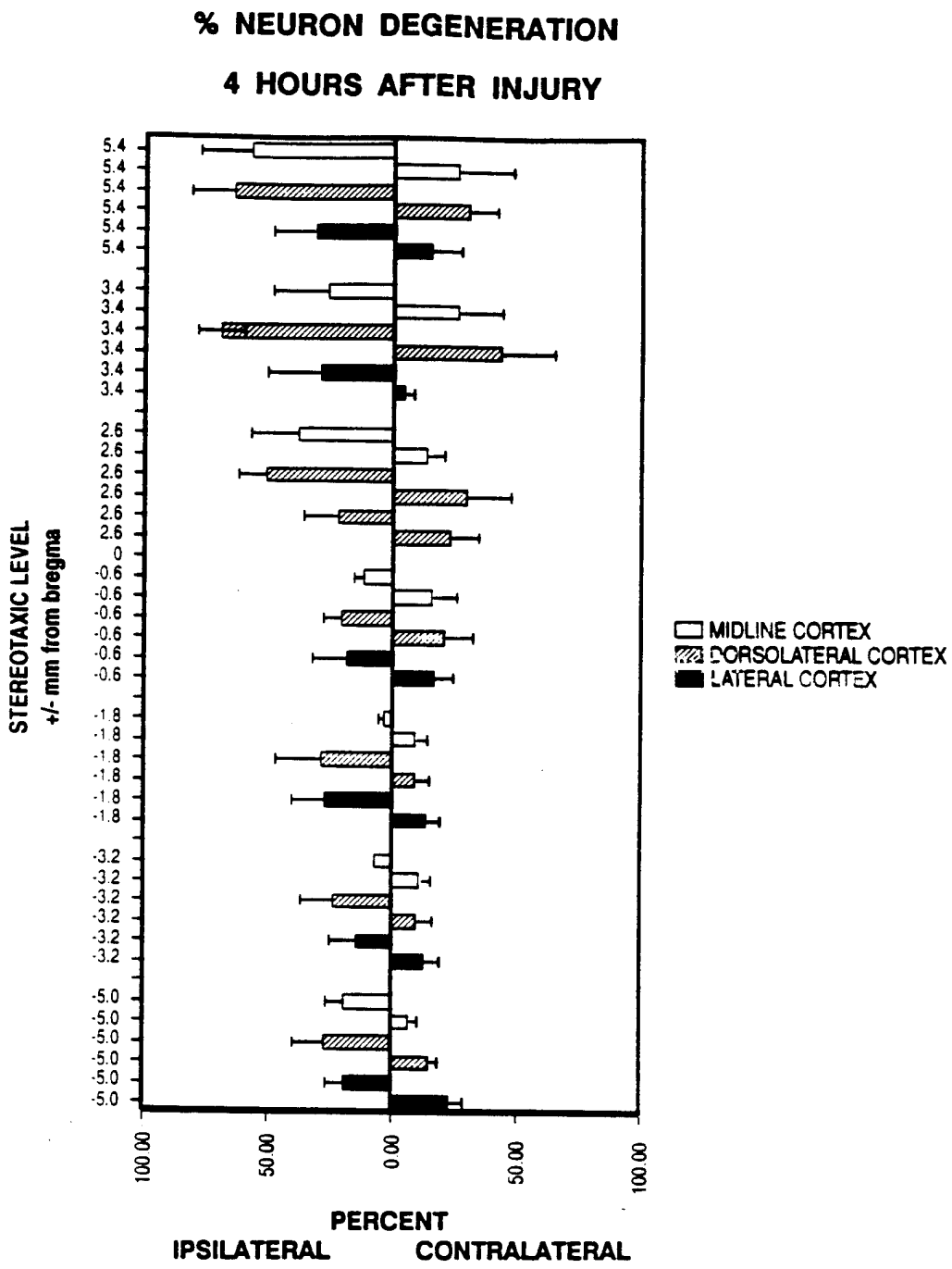


Figure 9A: Distribution of injured cells throughout the cortex for 4 rats four hours after injury. Injured cells were ubiquitous and did not predictably become less prominent in a graduated fashion with distance from the cortical impact site. Anterior regions had more injured cells than posterior brain areas. Data are means with SEM.

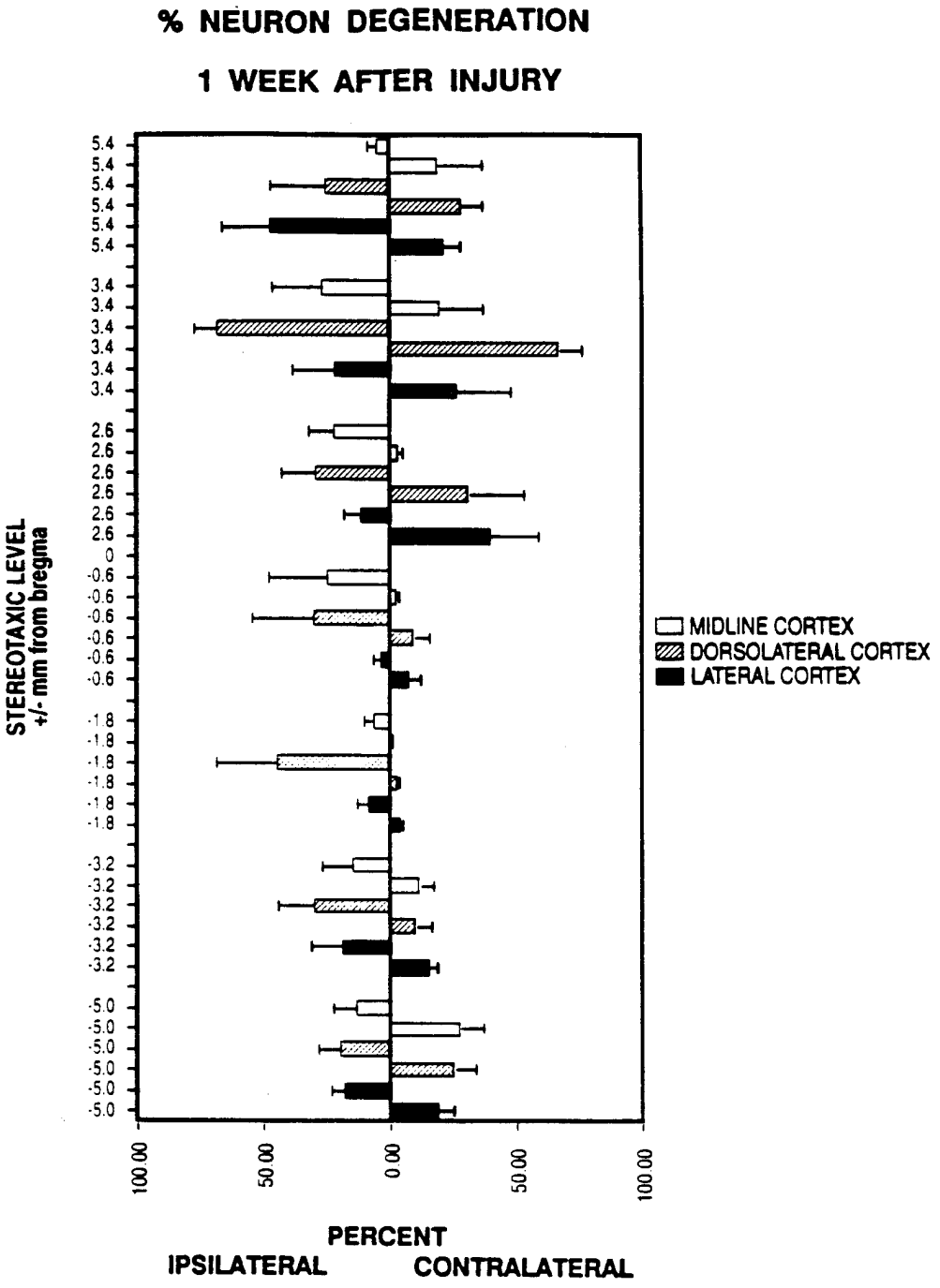


Figure 9B: Damaged cortical cells evaluated in 3 rats one week after injury. Again damaged cells appear in all brain areas. Data are means with SEM.

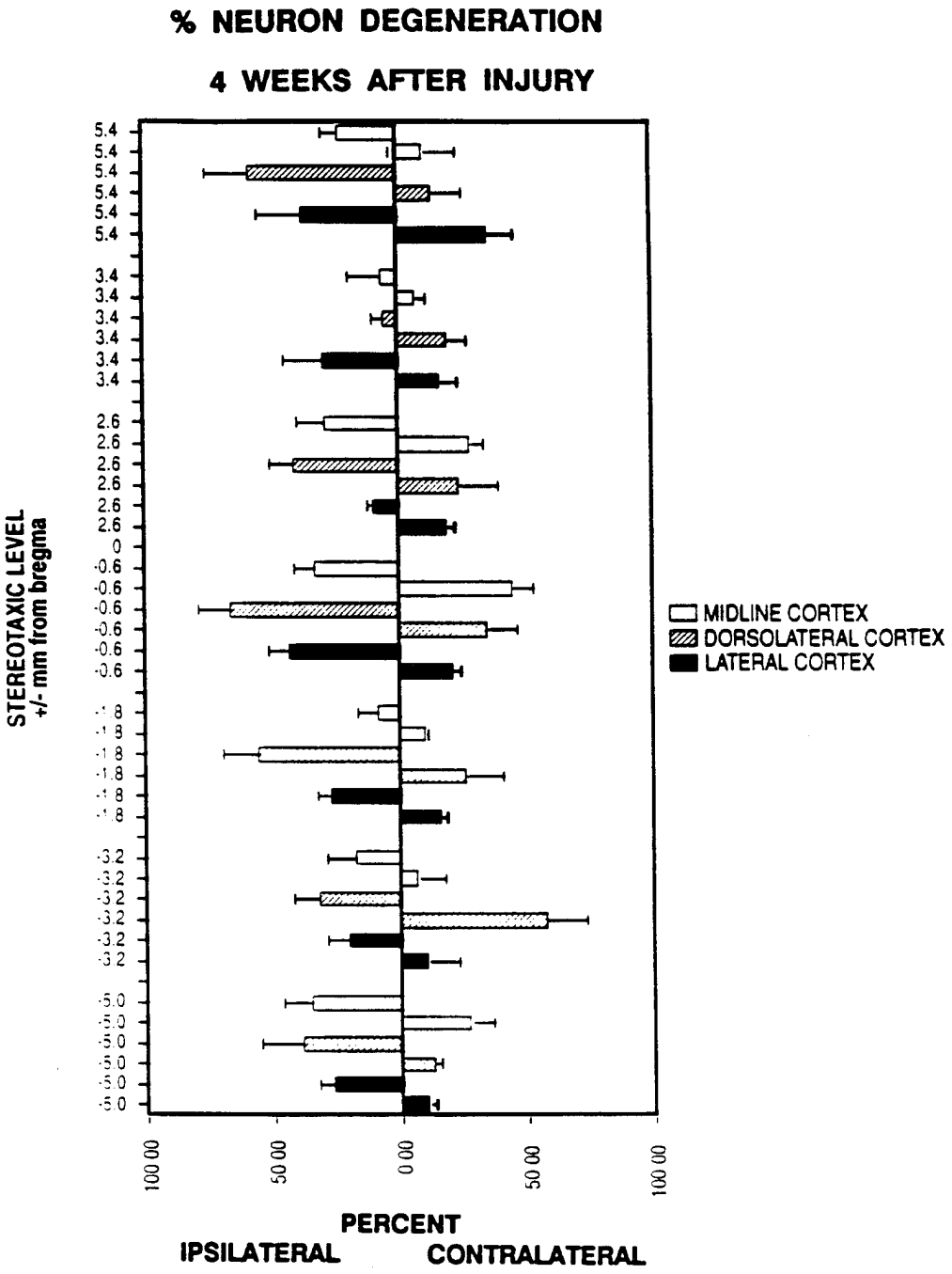


Figure 9C: Damaged cells from 4 rats. Even one month after injury some brain regions have more than 50% damaged cells. Data are means with SEM.

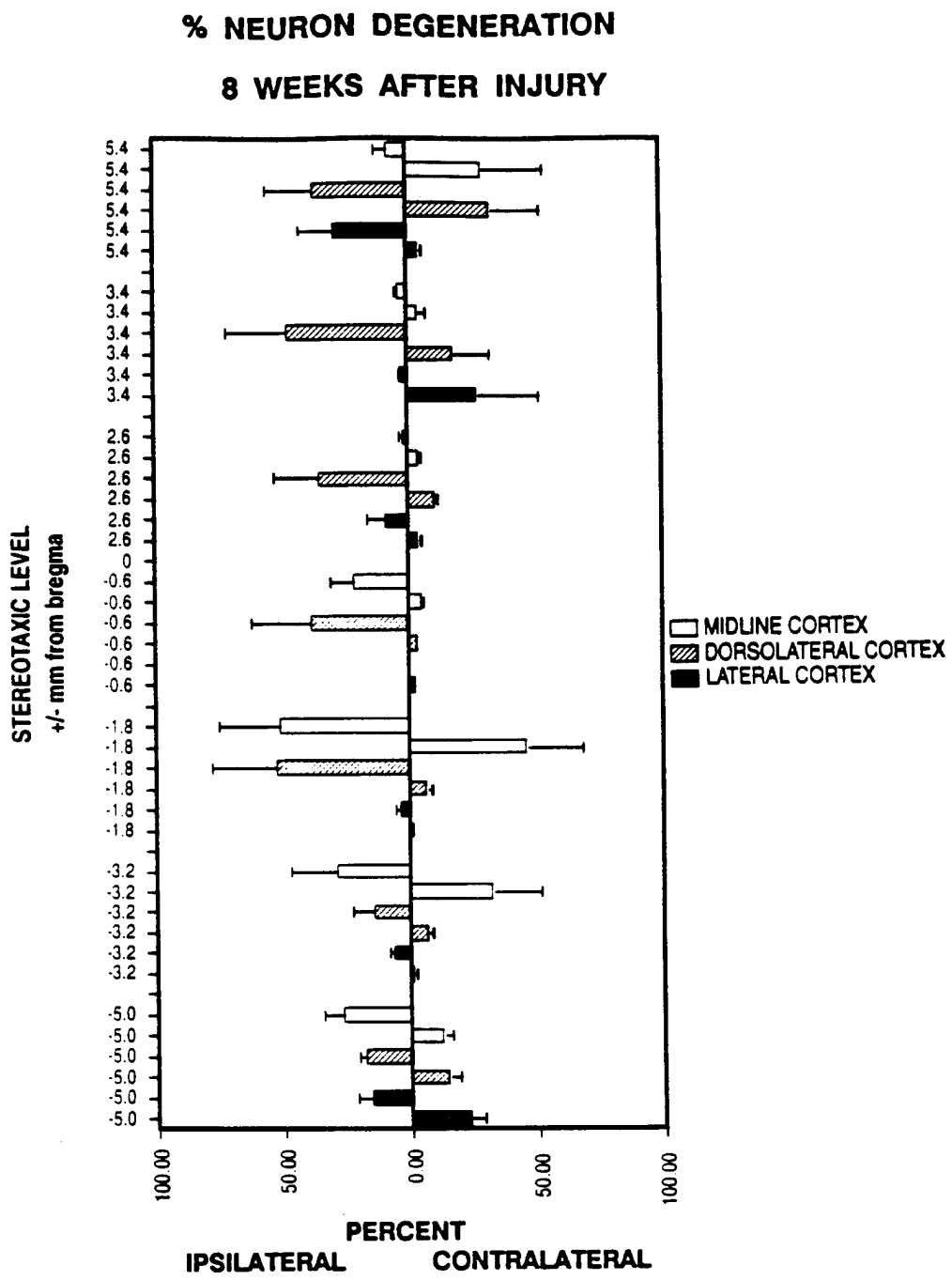


Figure 9D: Data from 3 rats suggest decreasing numbers of darkened (injured) cells. Disappearance of darkened cells could indicate recovery from injury and resumption of normal staining characteristics or ultimate cell death with disappearance of damaged cells. Data shown are means with SEM.

Discussion of Quantitative Cell Counts

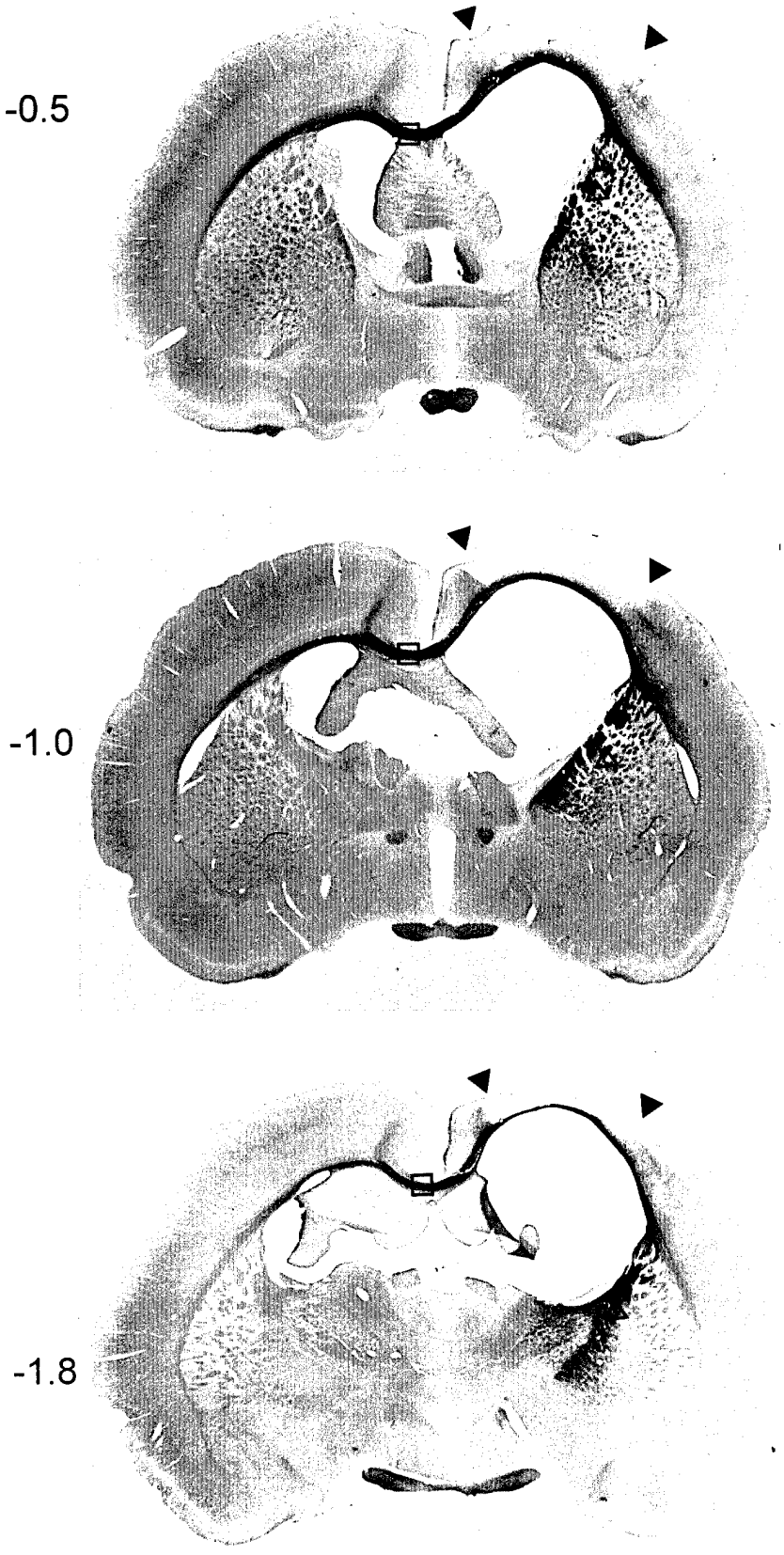
While the major effect of the piston injury involved the impact site, cellular changes indicating neuronal damage occurred very quickly and widely throughout the cerebral cortex. The percentage of abnormal cells decreased by 8 weeks. This could indicate either repair of damaged cells and the reappearance normal staining characteristics or death and drop out of the damaged cells. In either instance the number of darkened cells would diminish. The fact that ventricular dilatation occurs even on the contralateral side strongly suggests that the number of brain cells really does decrease widely throughout the brain after a focal impact injury.

The occurrence of widespread cell damage throughout the brain consequent to a supposedly focal injury in non ischemic or hypoxic animals may have importance in human head injury where the same widespread effects may be hypothesized. Widespread cell loss could explain soft or seemingly inexplicable neuropsychological findings or personality changes following focal brain injury. Traumatic brain injury has been known to result in widespread axonal shearing [58,59]. It has been suggested that such may lead to post traumatic loss of cerebral white matter. Our data suggest that not only may axons be sheared with traumatic brain injury but that neuron cell bodies themselves may be widely affected. **Parenchymal loss and subsequent ventricular enlargement may be the result not only of axon loss but diffuse cell body loss as well.**

C. Chronic (8 weeks) Studies of Fiber Track Axon Degeneration

We used the axon degeneration sensitive, cupric silver method of de Olmos [60] to demonstrate degenerating axons. An overview of the extent of axonal degeneration (darkened areas) is provided by figures 10A, 10B, 10C.

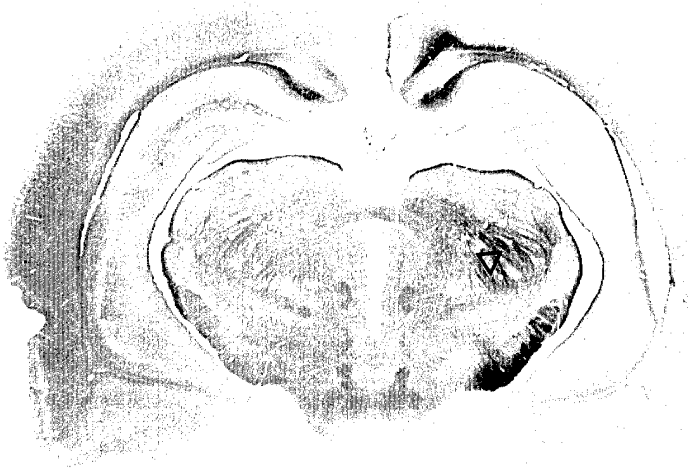
Figure 10a



▼indicates impact site. Note extensive degeneration (dark staining) in brain adjacent to injury, corpus callosum □ and striatum △.

Figure 10b

-4.0

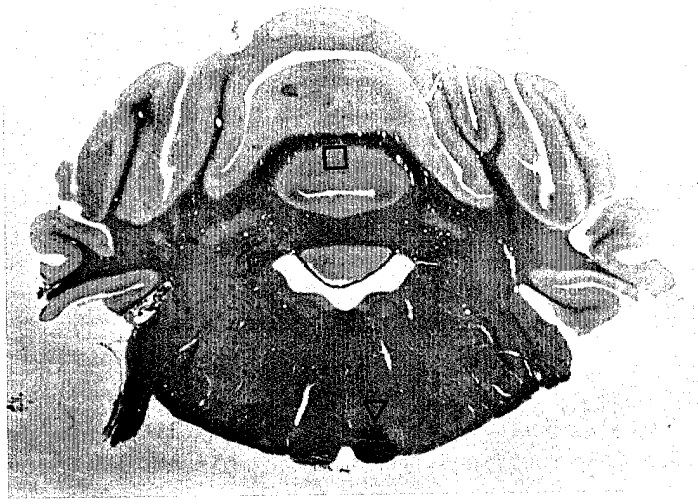


Degenerating axons are seen within the Thalamus ▽

-6.3

Degenerating axons within the Red Nucleus ▼

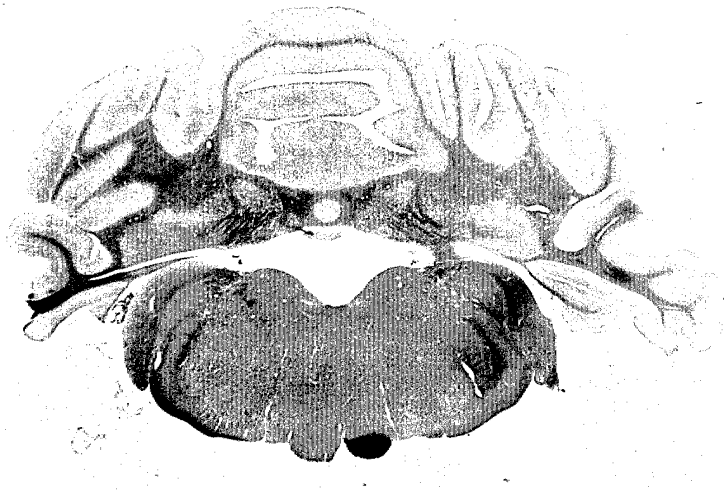
-10.5



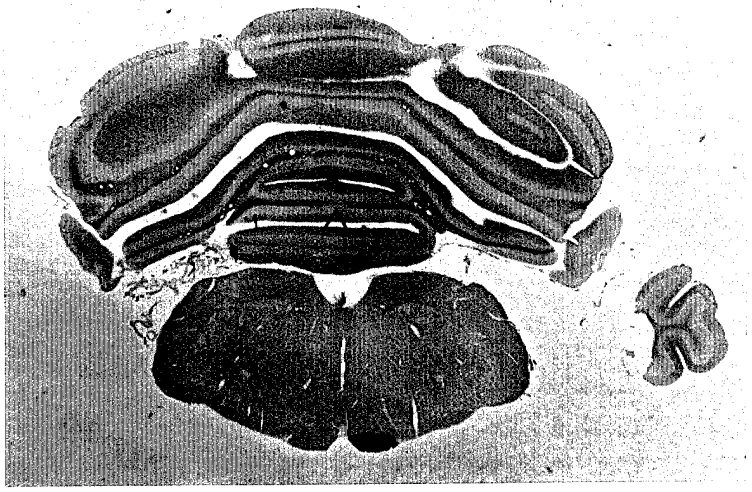
Degenerating axons within the Cerebellum □ and Corticospinal Track ▽

Figure 10c

-11.0

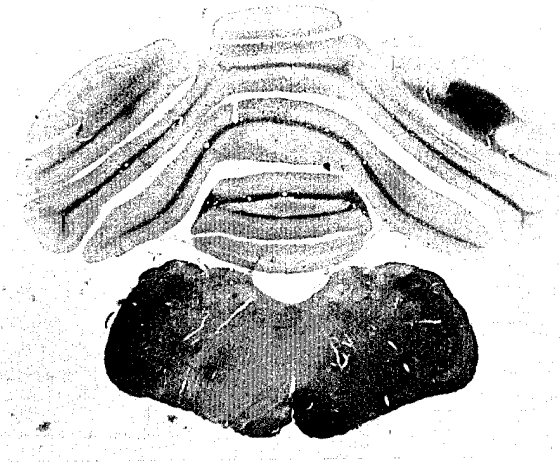


-12.0



Degenerating axons
within the Cerebellum △

-12.5



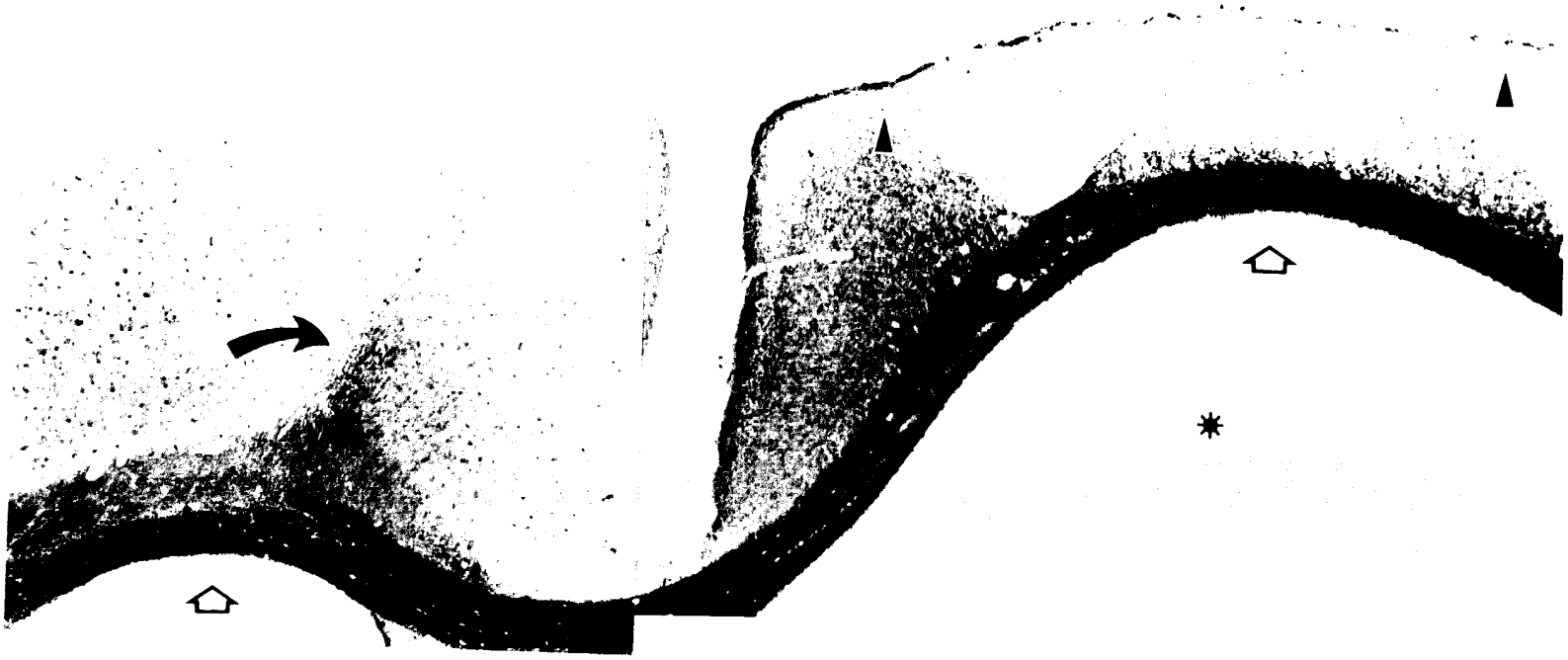


Figure 11. Ipsilateral (right) and contralateral (left) cortex. Solid arrowheads delineate the site of impact injury. The cerebral mantle is markedly decreased in thickness. Degenerating axons are abundant about the injury site. Note that the ipsilateral tissue about the midline (anteromedial and cingulate cortices) also appears darkened. Asterisk indicates enlarged ventricle. Open arrows point to darkly stained subcortical white matter which is continuous with the corpus callosum. The curved arrow indicates an example of degenerating fibers leading from the corpus callosum into the homologous area and the midline of the contralateral cortex. (30 X magnification).

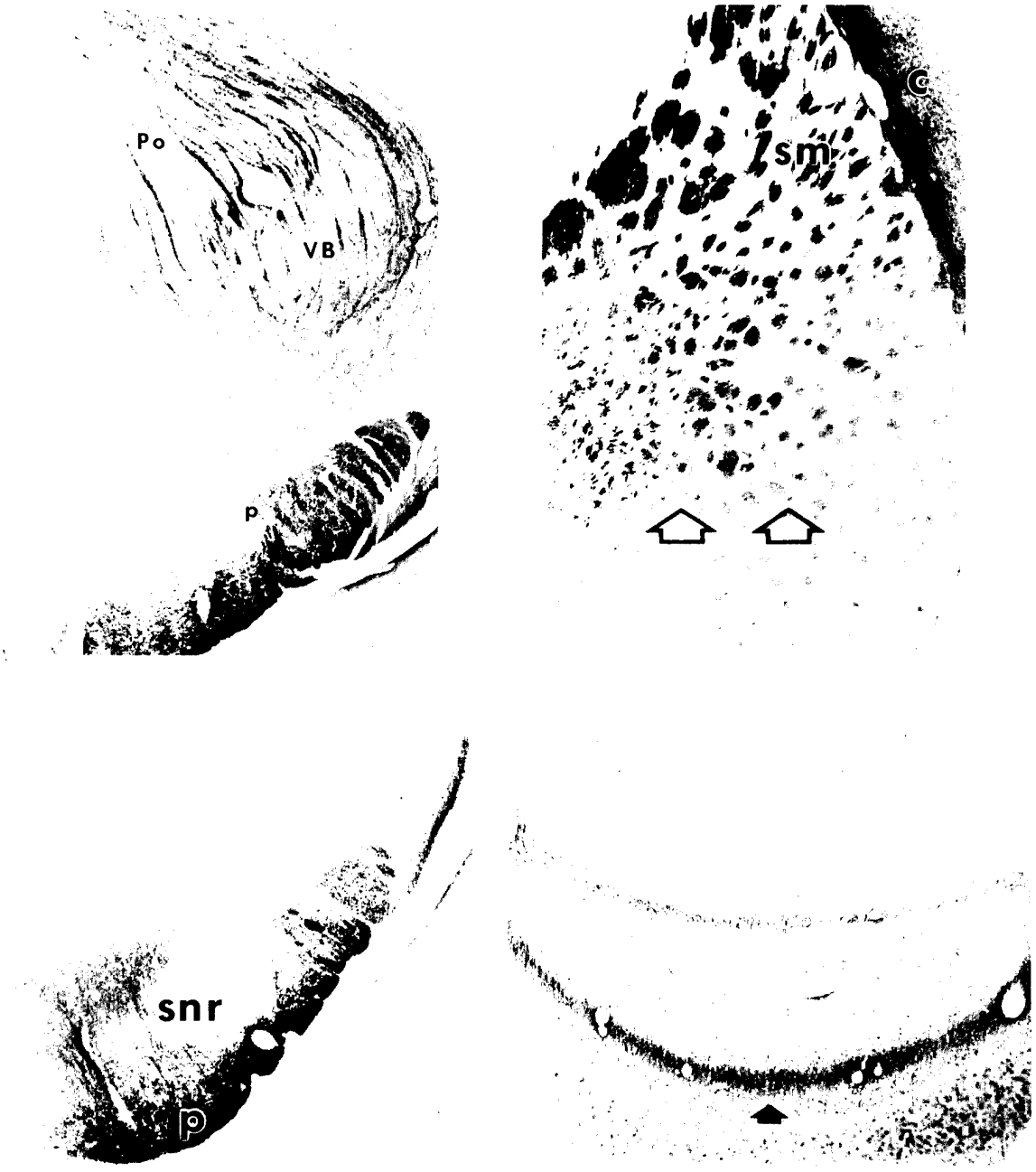


Figure 12. In the ipsilateral striatum (upper right) areas of axonal of degeneration are present in those areas shown to receive projections from the sensorimotor (sm) and limbic (l; medial mesocortex) areas [42]. Note that the area above the arrows appear darker than the areas lateral to and below the arrows indicating a topography of degeneration. C=cortex. The ipsilateral thalamus (upper left) contain degenerating axons in many nuclei especially the ventrobasal (VB) and posterior (PO) nuclei. Intensely stained axons also occurred in the ipsilateral peduncle (p). There were intensely stained axons passing from the peduncle (p) into the area of the ipsilateral substantia nigra reticulata (snr) (lower left). The stained fibers may be terminating in the nigral parenchyma or be sweeping up into the thalamus. The cerebellar vermis contained many degenerating fibers in the white matter (arrow) (lower right). (30 X magnification).

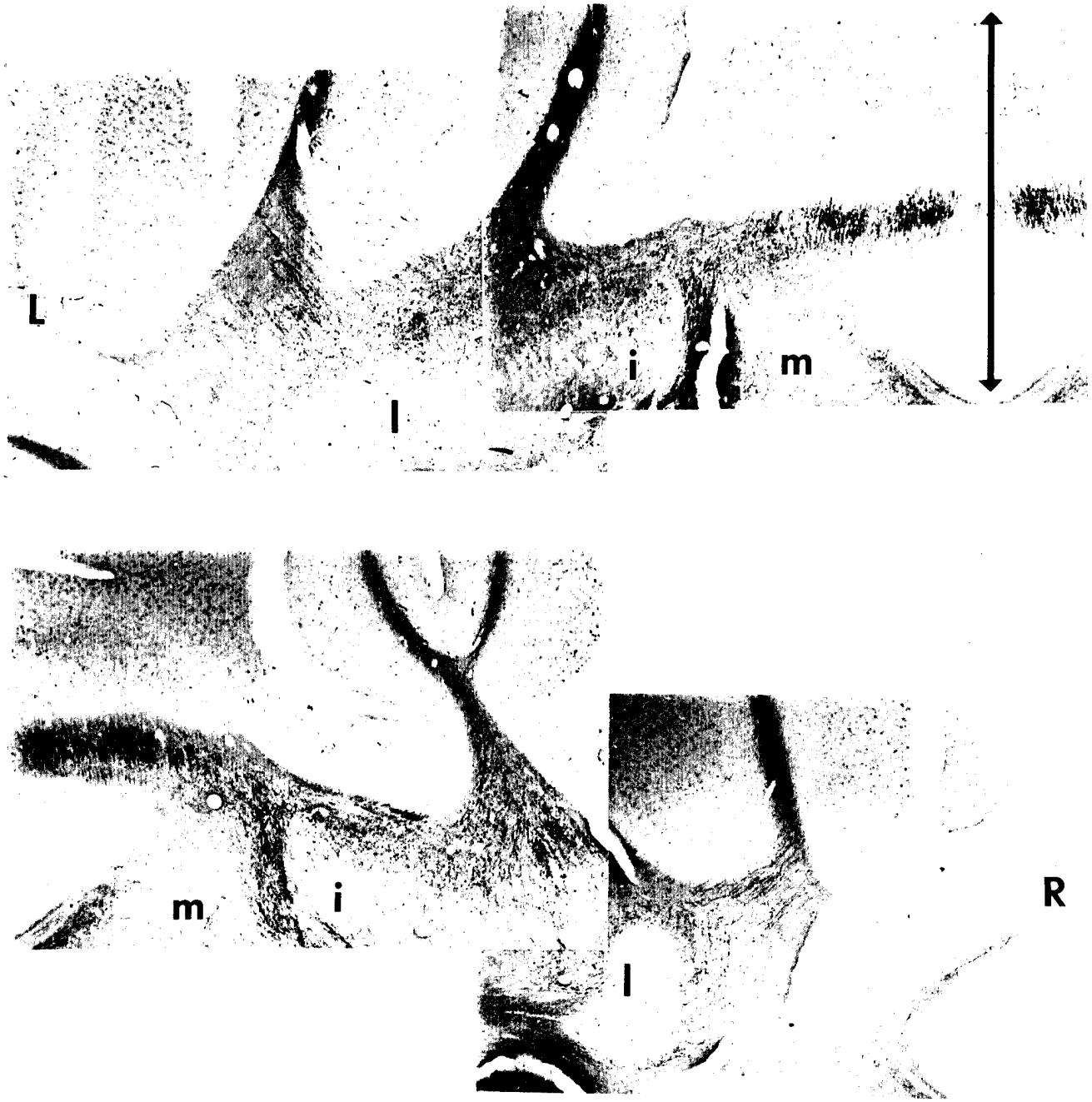


Figure 13. A montage of four micrographs encompassing the cerebellar white matter and adjacent folia extending dorsally and laterally from the midline (indicated by the double-headed arrow). The "L" and "R" delineate the left and right sides respectively. The medial (m), interposed (i) and lateral (l) deep cerebellar nuclei are indicated. Solid arrows point to silver-impregnated degenerating fibers which are widely distributed bilaterally in the white matter, extending into many of the folia and insinuated among the deep nuclei. The highest concentrations of degenerating fibers seem to occur most closely to the midline. (30 X magnification).

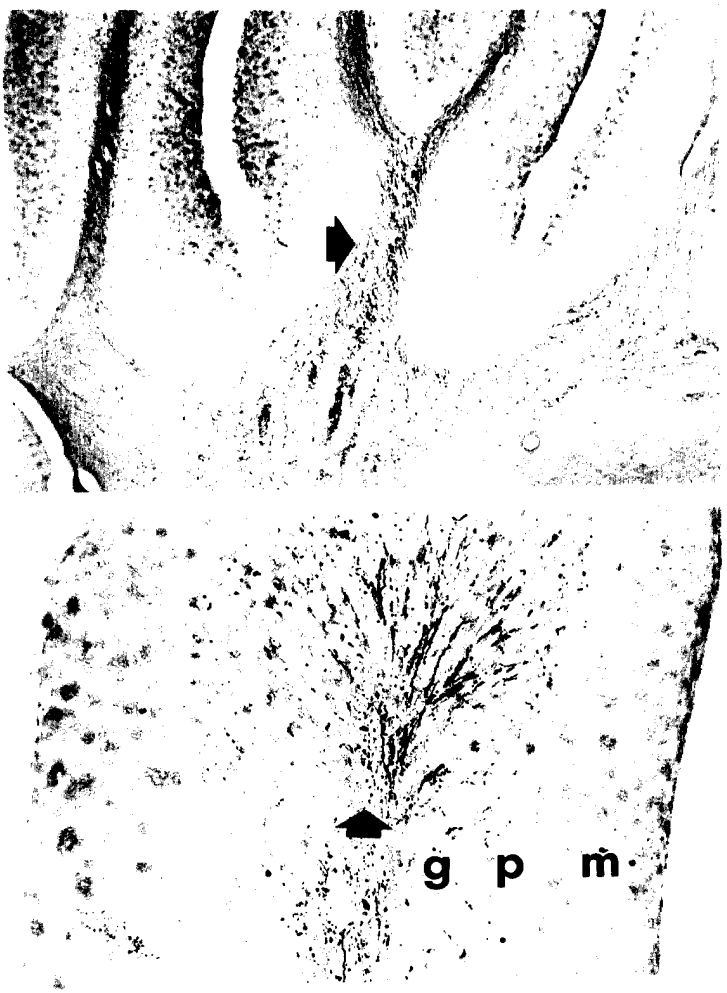


Figure 14. A photomicrograph which illustrates several cerebellar folia all of which exhibit degenerating fibers (arrow) (upper). (30 X magnification). At higher magnification (120 X) the granule cell (g), Purkinje cell (p) and molecular (m) layers can be seen (lower). Note that the degenerating fibers do not appear to extend any further than the granular cell layer.

Figures 11 through 14 provide detailed pictures of the axonal degeneration throughout the motor system. Degenerating axons were seen to extend from the cortex around the impact site in the adjacent residual right sensorimotor cortex. Degenerating fibers also entered the corpus callosum and projected across the midline to enter the contralateral midline and sensorimotor cortices, figure 11.

Large numbers of degenerating fibers were also seen to prominently descend into the ipsilateral dorsolateral and medial striatal areas. No contralateral striatal degeneration was observed, figure 12, (upper right).

In the thalamus axonal degeneration occurred in the ipsilateral ventroposterolateral (VPL) and ventroposteromedial (VPM) nuclei consistent with known connections between these nuclei and the forelimb and hindlimb sensorimotor cortices. Similarly, the axonal degeneration in the ipsilateral ventrolateral (VL), ventromedial (VM), ventrobasal (VB) and posterior (PO) nuclei is related to the connection of these nuclei with the ipsilateral motor cortices [61], figure 12, upper left). Axonal degeneration in the contralateral thalamic nuclei was negligible.

The ipsilateral substantia nigra displayed an extensive amount of silver-stained axons suggestive of possible projection of affected axons to nigral neurons, figure 12, (lower left). Negligible degeneration occurred in and around the contralateral substantia nigra. Degenerating fibers were seen in both red nuclei but more prominently ipsilaterally.

Unexpectedly, extensive bilateral axonal degeneration was seen in the cerebellum being especially prominent in the vermis, figure 12, (lower right). Degenerating fibers are also evident around the lateral, medial and interpositus nuclei, figure 13. Degenerating fibers were found to extend well into the folia and they appeared to terminate predominately in the granule cell layer, figure 14.

Discussion of Chronic Fiber Track Degeneration

Whereas degenerating axons in contralateral cortex, striatum, thalamus, substantia nigra and red nucleus would be expected owing to known commissural or projection fibers from the sensorimotor cortex, the extensive bilateral axonal degeneration in the cerebellum **present in every injured animal** was unexpected. This was not an artifact because no axonal staining occurred in control rat brains and the injured and control brains were mounted on the same slide and stained simultaneously. To our knowledge, such a pervasive effect of either traumatic brain injury or sensorimotor cortex lesions on axonal degeneration in the cerebellum has not been described before.

Presently it is difficult to offer an cogent explanation for this effect because there was no evidence of degeneration in either of the two primary afferent cerebrocerebellar connections: the pons or the inferior olive. **Possibly, the cerebellar axonal degeneration could have been caused by retrograde transsynaptic degeneration of cerebellar fibers projecting to the thalamus.** The lateral, medial and interpositus nuclei project to all the thalamic nuclei in which degeneration was observed (VPL, VPM, VL, VM, VB, PO) [61,62]. Similarly, the mossy fiber degeneration in the folia could have been the result of anterograde degeneration originating from the same thalamic nuclei.

We tentatively interpret these cerebellar findings as demonstrating transsynaptic degeneration in the cerebellum because direct sensorimotor cortex- cerebellar fibers are not know to exist.

Previous reports have indicated neurochemical cerebellar involvement after sensorimotor cortex lesions. For example, following sensorimotor cortex lesions in rats, infusions of norepinephrine into the cerebellum ameliorate beam-walking motor deficits [34]. Unilateral sensorimotor cortex injury caused decreased extracellular levels of norepinephrine bilaterally in rat cerebellum measured by norepinephrine turnover studies and by in vivo microdialysis [29,35]. These results were interpreted as showing a cerebellar diaschisis following sensorimotor cortex injury. The diaschisis was manifest by a general depression of the norepinephrine systems in the brain which temporarily inhibited a recovery of sensorimotor function [42]. Our data suggest that transsynaptic events including actual structural changes in cerebellar fiber tracks might play a role in these observations.

The entire subcortical motor system was widely affected by the cortical injury. While much subcortical degeneration could have been predicted by known cortical-subcortical connections (eg motor cortex-striatum; sensory cortex-thalamus) the possibility remains that transsynaptic degeneration occurred widely throughout the motor system and accounted for the florid axonal

changes seen in the entire motor system. **To our knowledge the possibility of widespread transsynaptic degeneration within the motor system has not heretofore been appreciated. Its existence has important ramifications not only for the occurrence of any neurologic deficits following sensorimotor cortical injury but for the recovery of neurologic function after injury and for brain plasticity. Perhaps neurologic dysfunction following brain injury can be ameliorated by preventing or reducing trans-synaptic degeneration occurring within the particular damaged functional system throughout the brain. Neuroprotective drugs may work at many sites within the brain not only at the site of injury itself but at subcortical levels subject to damage or functional alteration by transsynaptic degeneration. Perhaps plasticity and improvement of function after cortical injury involves not only reorganization of the cortex but of the entire subcortical functional system which has been subject to transsynaptic changes.**

Another possibility of course is that the observed cerebellar axonal degeneration was a direct effect of the cortical impact. The force of the impact, although mild, could have been sufficient to displace the brain within the cranial vault and cause the cerebellum to impact the surrounding skull resulting in neural injury. We feel this possibility is remote. This latter hypothesis is presently being evaluating by comparing the pattern of axonal degeneration following an electrolytic, non traumatic sensorimotor cortex lesion.

D. c-Fos Immunocytohistology

Introduction

c-Fos is a proto-oncogene, also known as an immediate early gene, which undergoes rapid but transient expression after cell stimulation. c-Fos, as is typical of all proto-oncogenes, encodes proteins that bind to DNA sites known to regulate gene expression. Regulation of gene epression may be a method whereby cellular stimulation brings about long term cellular changes [63,64].

c-Fos is contained in low levels in almost all cells in the nervous system and is readily expressed as a result of most types of cellular stimulation e.g. membrane depolarization, neurotransmitters, peripheral nerve input, and hormones. c-Fos is also believed to play a role in memory formation [63, 65-70]. As would be expected, pathological events such as seizures, cerebral ischemia and brain injury have also been found to induce c-Fos expression in adult nerve cells [71-82].

c-Fos expression induced by brain injury or cerebral ischemia is believed to be mediated by postsynaptic NMDA receptors, especially glutamate [75,82,83]. The NMDA subset of

glutamate receptors also regulates calcium entry into the cells. Calcium entry plays a role in cellular excitability and an excess of intracellular calcium causes cell death.

Therefore, we felt that using c-Fos immunocytochemistry following impact injury would provide an indicator of the neuronal response to injury throughout the brain. Then by using NMDA and AMPA/kainate receptor blockers we could also determine if the response was mediated by a NMDA glutamate receptor or by some other receptor.

Methods

After brain injury each rat was deeply anesthetized with sodium pentobarbital (50 mg/kg, IP) then perfused transcardially with a fixative consisting of 4% paraformaldehyde buffered with 0.067 M sodium cacodylate combined with 4% sucrose. The brains were removed and frozen in a slurry of dry ice and acetone. The frozen brains were mounted and brain sections (40 μm) were cut in a cryostat.

Brain sections were placed in immunocytochemistry incubation wells and washed three times in phosphate buffered saline (PBS) for 30 mins., then incubated in a blocking solution of 3% normal goat serum (NGS) for one hour on a shaker. After washing in PBS, the sections were incubated for 48 hours on a shaker in a 1:1000 dilution of rabbit anti-c-Fos protein generated against the N-terminal region of the c-Fos protein molecule (Oncogene Science). Sections were then washed three times (30 mins. per wash) in PBS and incubated in biotinylated goat anti-serum (Vector Laboratories) for one hour. After washing three times in PBS the the sections were incubated in avidin DH conjugated to biotinylated HRP (Vectastain ABC kit, Vector Laboratories) for one hour, rinsed three times in PBS and and washed three times in 0.05M Tris-HCL buffer (pH 7.6). The sections were then washed with 0.05% diaminobenzidine (DAB) in 0.05m Tris-HC1 buffer (10 mins) followed by a 5 min. wash of the same solution with 0.01% hydrogen peroxide added. The last 5 min. wash was along enough for the brown DAB reaction product to become clearly visible without interference from the background peroxidase reaction product. The sections were then washed with 0.05M tris-HCL (ph 7.6) and in PBS. One control section in each tray of wells was processed as above without exposure to the antibody.

Results

Cortical and hippocampal c-Fos expression was apparent 3.5 mins after injury, was widespread by 10 mins and sustained for up to 4 hours, figure 15A top. Although c-Fos activity was

intensely displayed in the dentate gyrus of the hippocampus, figure 15A bottom, not many darkened (injured) cells were found by light microscopy in this structure.

Figure 15A: Darkened cells indicate c-Fos activity in cortex (top) and dentate gyrus of hippocampus (bottom).

MK-801, a specific NMDA receptor blocker, administered at 3mg/kg (IP) was partially effective in preventing c-Fos expression as indicated by a reduced staining intensity in the hippocampus, figure 15B bottom. There was also reduced staining intensity in the cortex, figure 15 B top. When MK-801 was given at 1 mg/kg c-Fos expression was not prevented. The results indicate that a significant amount of c-Fos expression was probably mediated by the NMDA glutamate receptor subtype.

GYKI 52466, an AMPA/kainate receptor blocker, administered in a 10 mg/kg IV bolus followed by either a 60 mg/kg/hr or 15 mg/kg/hr IV infusion was minimally effective in preventing the c-Fos expression. This indicates that the c-Fos expression was not mediated by the AMPA/kainate glutamate receptor subtype (pictures not shown).

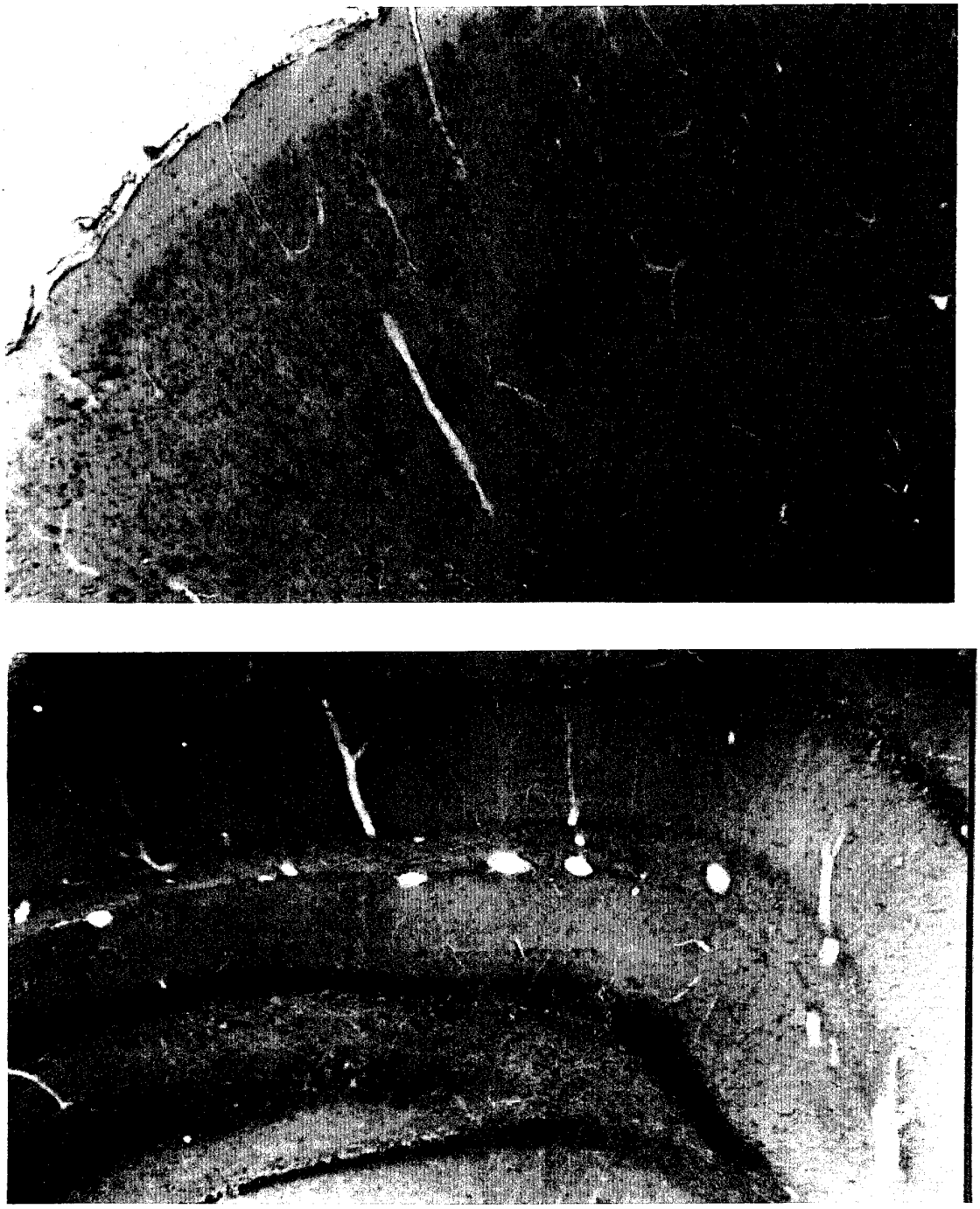


Figure 15B: MK 801, a specific NMDA receptor blocker, reduced but did not eliminate c-Fos activity in cerebral cortex (top) and hippocampus (bottom). c-Fos activity appeared more reduced in the cortex than the hippocampus.

Discussion of c-Fos immunocytohistology

The immediate appearance of c-Fos expression widely throughout the cerebral cortex after injury indicates widespread cellular stimulation. Possibly the widespread c-Fos activity could have been the result of spreading depression, a phenomena which can accompany brain injury and brain ischemia. Spreading depression is a pathophysiological, but non-lethal, disturbance of neural gray matter in which a transient depression of neural activity successively invades surrounding tissue. Spreading depression, which is thought to be mediated by glutamate via NMDA receptors, also causes great increases in cortical c-Fos activity. Thus, cortical c-Fos activity as we found in our rats, can merely represent brain injury-induced spreading depression [72,75,82].

Because cortical c-Fos activity was largely suppressed by MK-801, a specific NMDA receptor blocker, NMDA receptors would appear to be specifically activated by brain impact injury. The NMDA receptor activation could be the result of glutamate release which is known to occur after brain injury or cerebral ischemia [15,42,84]. Activation of the NMDA receptors has also been linked to glutamate induced cell death.

The presence of c-Fos expression in neurons, however, is not necessarily an early indicator of impending cell death but rather only that cell stimulation has occurred. Other methods are needed to determine if cell death has actually occurred concomitant with c-Fos activation. There is some congruence between the cortical areas showing increased c-Fos staining and cortical area containing darkened, injured cells. We tentatively conclude that the observed cellular injury and/or cell death seen in our quantitative cell counts may have been caused by excessive NMDA receptor activation, via glutamate release because MK 801 was able to strongly block cortical c-Fos expression in our rats. MK-801 has also been demonstrated to increase cell survival after cerebral ishemia [85]. However, we do not know if MK-801 would increase cell survival in our rats because we have not yet done cell counts in injured rats treated with MK-801.

We also found intense c-Fos activation in the dentate gyrus of the hippocampus but it was not correlated with significant cell darkening (injury or death) upon light microscopic inspection of the hippocampus. Possibly, the NMDA receptor activation was not sufficient to cause cell injury or death in this structure. In the dentate gyrus MK-801 was able to only partially attenuate the c-Fos activation indicating that some dentate gyrus c-Fos activation was not mediated by NMDA receptors. The small amount of c-Fos activation in the CA1, CA2 and CA3 regions of the hippocampus also did not appear to be NMDA

mediated since the c-Fos response was not attenuated by MK-801 in these areas.

Other investigators have noted that after fluid percussion injury there was an increase in c-Fos in the CA1 hippocampal area, but the increase was not correlated with injury severity or glutamate levels. These investigators hypothesized that after brain injury c-Fos expression was not a function of agonist surges alone but may reflect a more complex interaction of multiple receptors [80]. This c-Fos activation in the CA1 hippocampal area after fluid percussion injury contrasts with our injury model in which there was not significant c-Fos activation in the CA1 area of the hippocampus.

Based on a review of literature and our results, we believe that greater use of c-Fos analysis in our research may not be justified at this time. When we first wrote our proposasl in 1991, c-Fos analysis appeared to have a greater potential than has been demonstrated over the past 4 years. For instance, there is no precise meaning of brain c-Fos expression making c-Fos results difficult to integrate into important research questions and decisions for future research. Early on it was assumed that c-Fos expression may be an indicator of neuron degeneration sites after brain injury [75,76]. Evidence since that time indicates that c-Fos expression may be indicative of a neuroprotective process because c-fos expression has been shown to precede both nerve growth factor (NGF)-mRNA and NGF-like proteins in the cortex after spreading depression [77]. Thus c-Fos expression after brain injury can only be used as an indicator of where cells may die but c-Fos by itself does not indicate cell death is certain to occur. To determine whether cells exhibiting c-Fos activity actually died it would be necessary to perform quantitative cell counts to see whether cell populations actually did decrease.

We have recently started to use silver staining techniques which appear to be more informative in regards to cellular states and as an indicator of where to conduct neurochemical analyses. The silver stained brain slices depict areas where actual axonal degeneration has occurred. We have presented numerous pictures in this report (section 2C. pg. 26) which illustrate axonal degeneration throughout the motor system in response to sensorimotor cortex injury. The silver staining technique will be invaluable for our upcoming drug tests since we will be able to determine whether a drug treatment results in attenuation of axonal degeneration. Axon integrity, in turn, requires a healthy neuron cell body.

3A Neurobehavioral Studies (Dr. Soblosky)

Motor Deficits

We felt that brain injury involving both fore and hindlimb cortical areas would be the most clinically meaningful because many brain injured people have a hemiparesis. Furthermore, Dr. Soblosky felt that a battery of 4 tests to evaluate both fore-and hindpaw function after injury would be more powerful than using a single test. The four tests chosen were: 1) the beam walking task which evaluated the "style" in which the rats traversed the beam after injury. This test's neurologic score proved excellent in evaluating both fore and hind limb deficits. Although the rats attained the ability to place their hindpaw on the horizontal surface of the beam and easily cross it after injury, they tended to have two or more toes protrude off the beam. This minor but significant degree of toe misplacement, not usually exhibited before injury, accounted for most of the recover delay documented by this test. 2) The pegged beam test evaluated both forelimb and hindlimb sensorimotor functioning as well as right side and left side hindlimb/forelimb coordination. Paresis of both left fore and hindlimbs may have exacerbated the difficulty of this task because the rat could not use its ipsilateral forelimb to help a dysfunctional hindlimb move around a peg as do rats with only a hindlimb deficit (unpublished observations). This inability to compensate for left sided hindlimb locomotor deficit accentuated the number of left hindlimb foot slips. 3) The number of left forepaw foot faults on a wire grid measured primarily forepaw sensorimotor function. 4) The forepaw preference test measured forepaw asymmetry and did not require pre-training or testing in an "unnatural" situation. Rather, this test measured how the cortical injury affected each rat's normal use of a forepaw for support, rearing and landing. Some tests could not be completed by brain injured animals until several days after injury.

Methods of Neurologic/Behavioral Evaluation

1) Beam Traversing

Rats were pre-trained to traverse an elevated narrow wooden beam (2.5cm wide X 120cm long x 69cm high) using white noise (60dB) as an aversive stimulus. Rats were negatively reinforced by termination of the white noise upon entering a goal box at the end of the beam. Motor performance was scored by a system adapted from Feeney et al [42] modified to measure forelimb as well as hindlimb deficits (See Appendix A, Table 1).

Each animal was scored by two observers one of whom was blind as to the injury status of each animal.

2) Foot-slips While Traversing a Narrow Pegged Beam

Rats were pre-trained to traverse an elevated narrow beam (2.5cm X 120cm X 69cm high) which had 4 steel pegs (2mm diameter X 3cm high) equally spaced in an alternating sequence along the beam [36]. Pre-training was conducted as for the non-pegged beam. The number of foot-slips (both hind and forelimb) made while traversing the pegged beam were counted by two observers one of whom was blind as to the injury status of each animal.

3) Left Forelimb Foot Fault Testing

Rats were placed on an elevated grid surface (23.5cm wide X 43.5cm long X 92 cm high; 2.5cm square/grid opening, 2mm diameter grid wire) [86,87]. Over a three minute test period each rat's ambulatory activity over the grid was recorded on videotape while the number of left forelimb foot faults for each rat were counted. The total number of forelimbs steps was determined from the videotape and the data expressed as the percentage of left limb foot faults i.e.

$$\left(\frac{\text{number of foot faults}}{\text{total number of forelimb steps}} \right) \quad X\ 100$$

4) Forelimb Paw Preference Test (Forelimb Asymmetry)

Rat were placed in a clear plastic box (inner dimensions: 10.3cm wide X 30.5 cm long X 38.5cm high) and their activity videotaped for 5 minutes. From videotape playback, instances of asymmetrical forelimb use were recorded which included observations of 1) the limb used to push off the floor prior to rearing, 2) the limb used for single forelimb support on the floor of the box, and 3) the limb used for single forelimb support against the walls of the box [88,89]. Data were expressed as percentage of right (unaffected by injury) forelimb use i.e.

$$\frac{\text{right forelimb use}}{\text{right + left forelimb use}} \times 100$$

Results of Neurologic/Behavioral Evaluation

Beam Traversing on a Non-Pegged Beam

Beam traversing was significantly impaired in injured rats up to 56 days post-injury (U=O, p <.001, Mann-Whitney U-Test), figure 16, pg 47.

Foot-slips on a Pegged Beam

Brain injured rats had more footslips than control rats ($F_{1,20}$ = 79.43, P<.001) but the number of footslips decreased with time after injury ($F_{10,200}$ = 13.76, p<.001) There was also a time X injury interaction ($F_{10,200}$ = 10.68.68, p<.003). Since the time at which individual rats were able to complete the pegged beam test varied, specific comparisons could only be made beginning at 5 days post-injury. Specific comparisons indicated that the injured rats had more footslips than control rats at all time points up to 28 days post-injury (all P<.05), figure 17, pg 48.

Left Forelimb Foot Faults

Injured rats had more left paw foot faults on the wire grid than control rats ($F_{1,20}$ = 101.52, P<.0001) and the number of foot faults decreased over time after injury ($F_{10,200}$ = 17.13, P<.0001). There was an injury X time interaction ($F_{10,200}$ = 12.30, P<.0001). Specific comparisons indicated that the injured animals had more

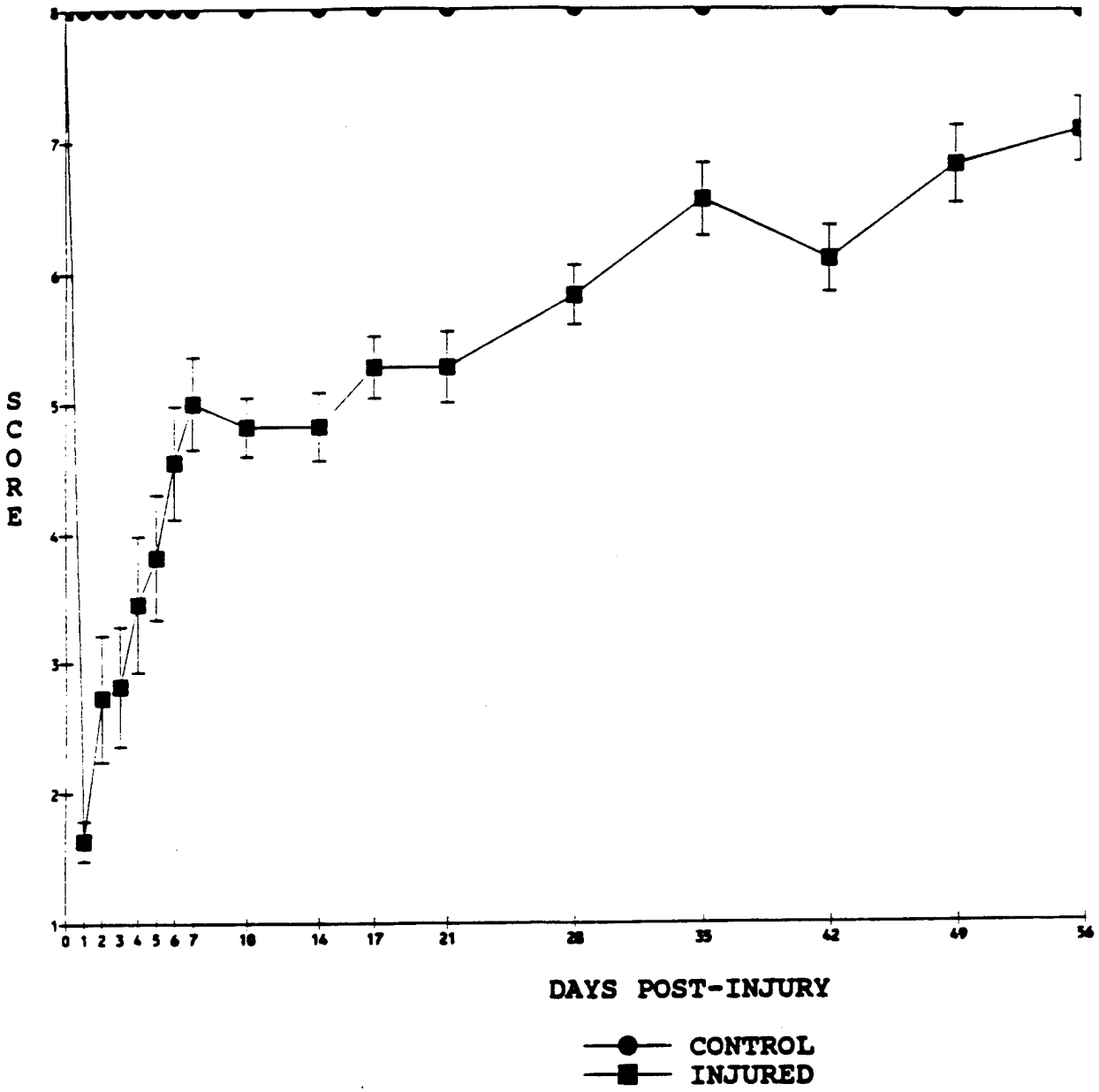
left forepaw foot faults than control animals up until 28 days post-injury (all P<.05) Measurements taken at 21 days post-injury, however, were not statistically significant, figure 18, pg 48.

Forelimb Preference Test

Before injury the rats to be injured did not differ from the control rats in their forepaw bias ($F_{1,20}$ = .19, P =.67). After injury rats displayed a high bias to use their right, unaffected paw as compared to controls ($F_{1,20}$ = 58.97, P<.0001) but this bias decreased with time after injury ($F_{10,200}$ = 7.47, P<.0001). There was also an injury X time interaction ($F_{10,200}$ = 7.80, P<.0001) and specific comparisons indicated that the injured animals displayed a bias to use their right (unaffected) paw as compared to the control animals up until 35 days post-injury (all p<.05), figure 14, pg 49.

Figure 16

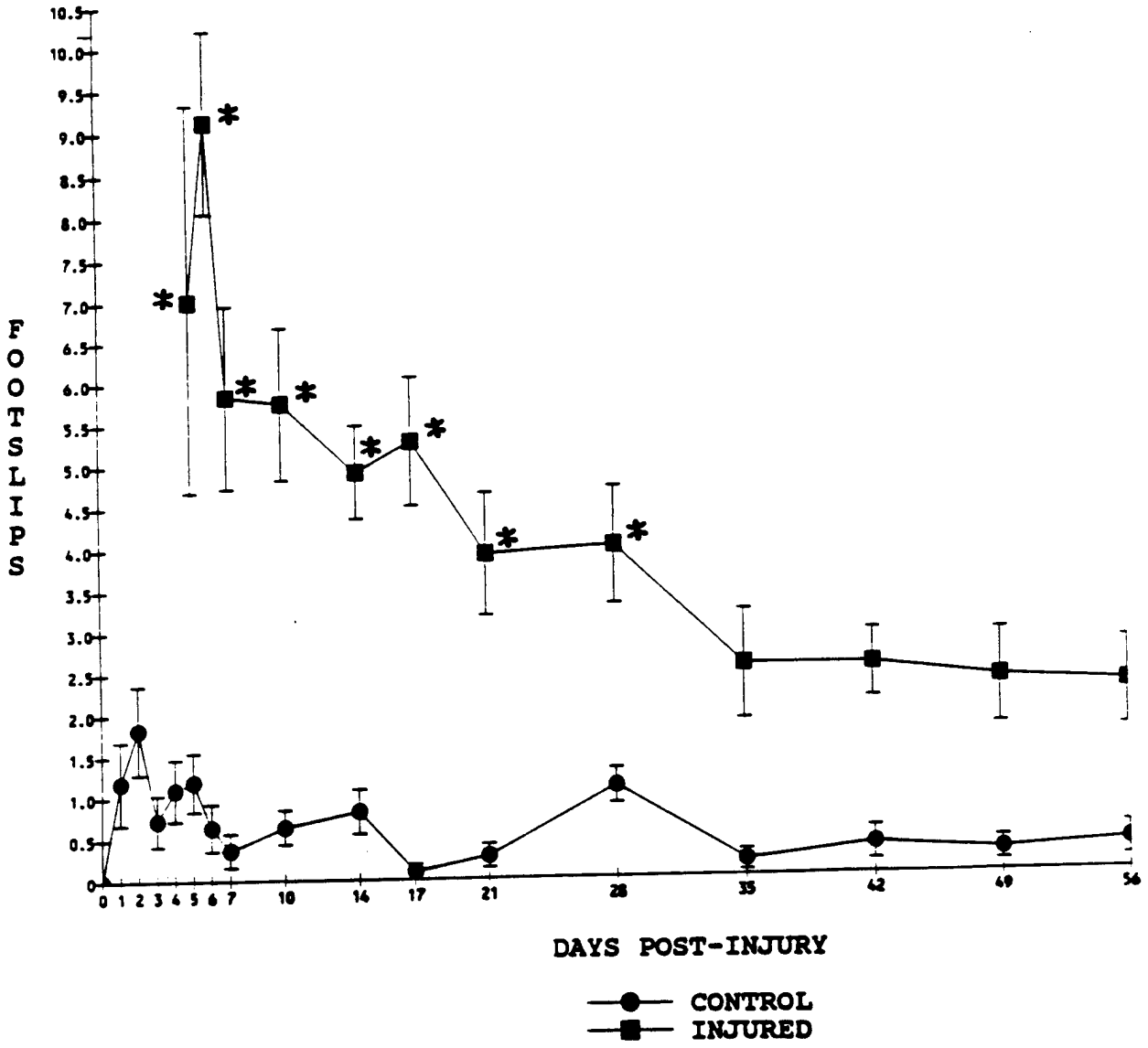
NEUROLOGICAL SCORE ON BEAM WALKING ABILITY AFTER BRAIN INJURY



N=11 per group. Injured animals differed in recovery rate over the 56 day period post-injury ($p<.001$).

Figure 17

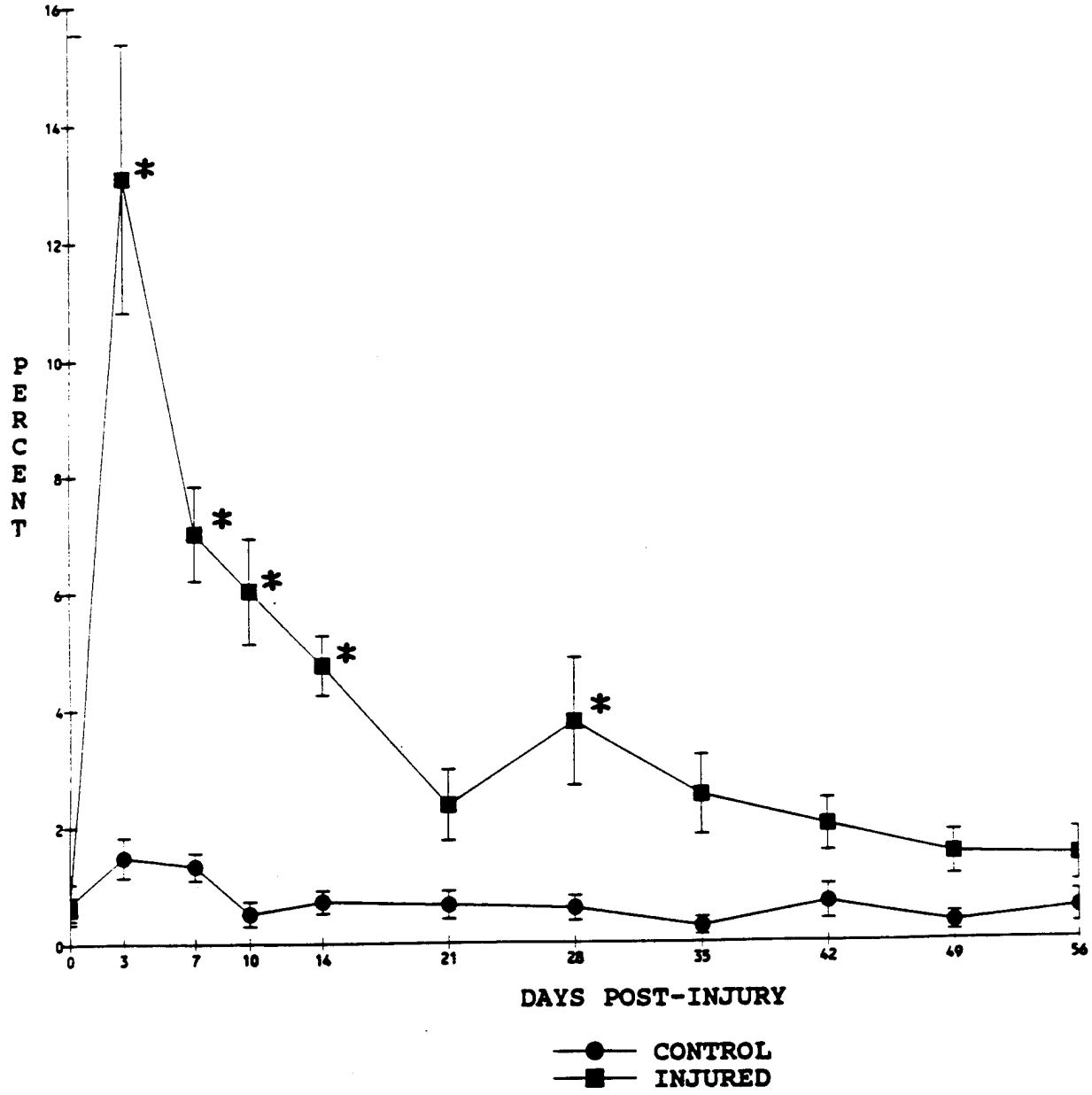
NUMBER OF FOOT SLIPS ON PEGGED BEAM



Injured animals data begin on Day 5 because on Days 1 and 2 post-injury no rats could traverse the beam. On Days 3 and 4 only one and two rats respectively could traverse the beam. On Day 5, n=6; on Days 6,7 and 10, n=8. N=11 per group. * = $p<.05$ vs controls Bonferroni test.

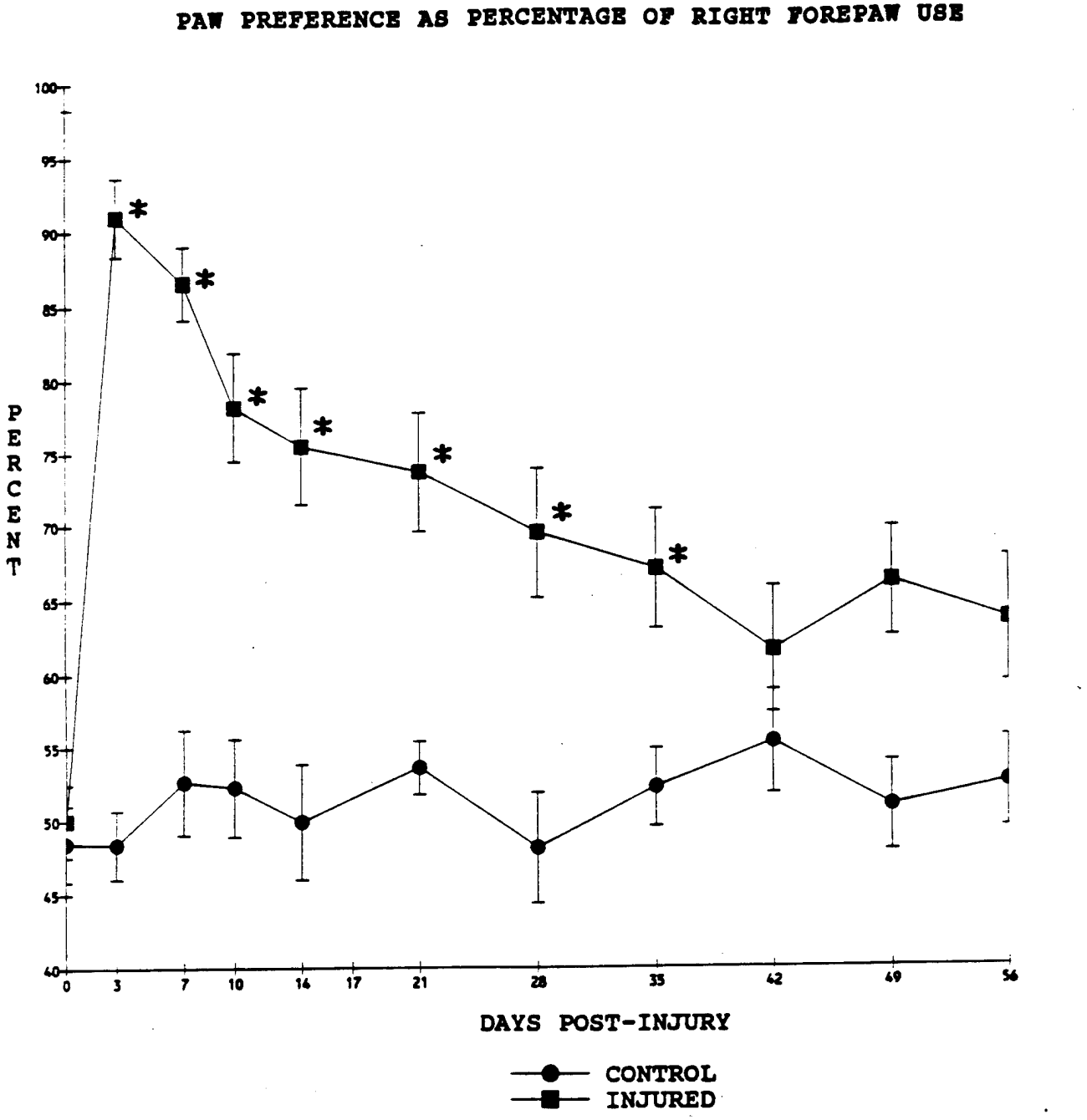
Figure 18

PERCENTAGE OF LEFT FOREPAW FOOT FAULTS AFTER INJURY



Injured animals made more left forepaw foot faults on the wire grid for 28 days after injury. This results indicates a slow recovery/adaptation for the forepaw deficit. n = 11 per group. * = p<.05 vs controls.

Figure 19



Injured rats showed an extreme preference to use their right forepaw (uninjured) for 35 days after injury. Rats do not normally show asymmetric forepaw usage during "natural" behavioral movements as evaluated in this test. n=11 per group. * = p<.05 vs controls.

Discussion of Motor Deficits Following Traumatic Brain Injury

These tests showed excellent sensitivity to the motor deficits induced by our injury model. This four test battery injury documented a longer time for improvement of hemiparesis (28-35 days) than has been customarily noted in the hindlimb deficit model which often shows improvement to normal status in as little as 5-12 days when determined by beam traversing and beam balance tests [4,8,34,42,90]. Documentation of a longer time to reach significant behavioral improvement is beneficial because if such improvement occurs too quickly there may be too short a time in which to test drugs to determine possible efficacy following brain injury.

When first tested after injury the rats exhibited severe deficits (figures 16 to 19). Improvement occurred initially over the first 7 to 14 days, followed by slower improvement lasting many weeks. Several interpretations of this biphasic recovery curve are possible: 1) possibly the period of early rapid improvement reflects resolution of the acute pathobiologic processes consequent to brain injury (e.g. decrease in local brain edema or the effects of excitotoxic amino acids, free radicals etc.) which potentiate actual neural injury consequent to the trauma [20,91,92]; 2) possibly the rapid recovery phase occurs consequent to the early functional return of less severely damaged cells around the periphery of the impact site; 3) possibly early rapid recovery occurs because other brain areas which normally participate in the motor functions along with the damaged cortex quickly begin to reorganize and take over some of the functions of the damaged cortex. Brain lesion studies which evaluated skilled forelimb use in the rat have suggested that several brain areas (e.g. caudate-putamen, globus pallidus) may be involved in sensorimotor improvement following injury [93].

Although the actual processes involved in functional recovery of are still unknown, **our data indicate the importance of behavioral compensation/adaptation, especially in the early phase (days) after injury.** Toe misplacement on the beam walking task suggests that true neural recovery had not occurred and that compensation/adaptation was responsible for the rats' ability to place their hindlimb on the horizontal surface of the beam. This interpretation is further supported by the results obtained from the pegged beam test which unmasked the persistent hindlimb sensorimotor deficit (many footslips) in rats which were able to maintain their hindlimb on the horizontal surface while crossing the non-pegged beam. Over time the rats significantly improved even on the pegged beam but improvement on this test always lagged behind the non-pegged beam (figures 16 and 17) Clearly no cross transfer of compensatory movement from the non-pegged to

the pegged beam occurred since the rats "recovered" hindlimb deficit as tested on the non-pegged beams (except for toe misplacement) reappeared on the pegged beam. Even improvement on the pegged beam could be the result of compensation/adaptation. The hindlimb was not further evaluated with yet another test in order to challenge the apparent "recovery" on the pegged beam. (Indeed there could be no end to such further testing and retesting). Forelimb placements on the wire grid also improved after injury but the rats appeared to have altered their style or strategy in searching for the grid wires. They used quicker and shorter repetitive forelimb movements to search for a wire to engage. As the rats became more proficient in this strategy forelimb placement continued to improve. In the paw preference test it could be argued that using the unaffected forelimb for supporting and rearing activity is obviously compensation/adaptation.

Further evidence from other laboratories which supports behavioral compensation/adaptation as dominating the early stages of "recovery" would include the enhanced recovery rate (hours) from a hindlimb deficit seen on beam traversing tests after amphetamine administration or by merely prodding the rat to cross the beam [42,94]. These researchers interpreted the enhanced recovery in their animals as being caused by restoration of injury-induced depression of noradrenergic functioning (i.e. diaschisis). Perhaps restoration of noradrenergic functioning by drug treatment plays a key role in enhanced behavioral compensation/adaptation since the deficits can be made to reappear by giving drugs which block noradrenergic functioning [30].

The later phase of behavioral/neurologic recovery may reflect actual restoration of damaged cells and/or the further reorganization of ipsilateral and/or contralateral neural circuits. Actual neural changes in the sensorimotor cortex concomitant with behavioral (sensorimotor) changes have been recently described in adult rats [88,89,95]. As already mentioned the cerebellum has also been implicated in recovery following sensorimotor cortex injury because norepinephrine infusions into the cerebellum contralateral to the injury enhanced recovery [34]. The cerebellum undergoes synaptogenesis as a result of motor learning, but not with random activity [96]. Possibly the extended practice of compensatory movements following brain injury may induce cerebellar or other brain area(s) synaptogenesis necessary for a true neural recovery.

While these various mechanisms are only theoretical possibilities at this point, the greatly differing recovery slopes early and later after brain injury observed in our rats

suggest that different processes account for early and late recovery. It may be that classes of drugs which would be effective early after injury would be ineffective in the later phase of neural recovery and visa versa because different biological processes may be affected.

It has been shown that a single drug can produce different effects depending upon whether it is given before or after injury. For instance, MK-801 given before sensorimotor cortex injury is beneficial, but if given several days after injury, it may impair recovery [97]. Similarly, rats given MK-801 before brain injury did not show cognitive impairment in response to MK-801 given several days after brain injury, while rats which did not receive MK-801 before brain injury displayed cognitive impairment when given MK-801 several days after brain injury [98]. Perhaps a more clinically relevant test for a single drug would be to test the effect of the drug given shortly after the injury (10-120 mins) versus its effect when given when given several hours or days after injury.

What is actually being measured in animal "recovery" is a vexatious issue [99-101]. The key problem involves actual neural recovery versus behavioral compensation or adaptation. True neural recovery would entail the complete return of pre-injury neural function and style in the affected limb while behavioral compensation or adaptation does not necessarily require actual return of the lost neural function and results in an altered style or strategy. It may be difficult to ascertain the precise contribution of compensation/adaptation and true neural recovery in the improvement in impaired performance following brain injury. Nevertheless, if impairments caused by brain injury are going to be ameliorated by pharmacologic or other therapies it would be important to determine whether the end result represented behavioral compensation or a true neurologic recovery. This issue has been addressed in regards to the beneficial effects of environmental enrichment by Rose et al 102,103] who contend that most studies claiming to demonstrate environment enrichment-induced recovery are actually evaluating behavioral compensation.

By whatever means "recovery" occurs, to show that a specific function has fully recovered, a well designed test battery with cross validation of the observations would be more powerful than any single test. Any apparent recovery shown in one test could be confirmed by another test. The second test should evaluate the specific behavioral deficit by a different criterion so that any compensatory movements or strategies acquired from the first test would have little chance of being cross transferred to the second test. Even using a test battery, however, it may be very

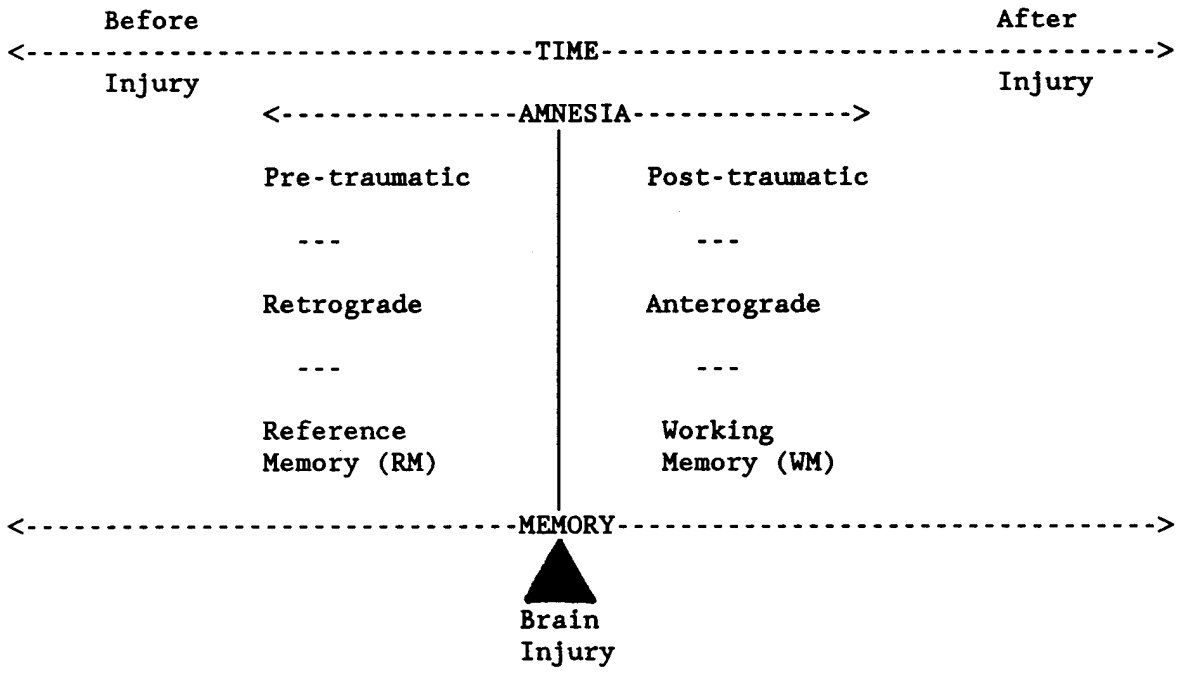
difficult to ultimately dissect out compensation/adaptation from true neural recovery.

Although true neural recovery after brain injury may be only partial, the ability to acquire associated compensatory movements is also important especially as the acquisition of compensatory movements may also result from neural recovery or reorganization mechanisms. Any drug or other therapy which aids either neural recovery or promotes the acquisition and maintenance of compensatory/adaptive movements would be invaluable.

3B Neurobehavior Studies (continued)

Memory Deficits

Traumatic brain injury in humans often results in cognitive disturbances and learning and memory deficits [104]. Memory deficits associated with concussion may be manifested by failure to recall events occurring prior to or after brain injury. Memory loss for events occurring before is called retrograde or pre-traumatic amnesia. Failure to recall events after brain injury is called anterograde or post-traumatic amnesia and is felt to more closely correlate with the severity of the brain injury. Anterograde amnesia also affects learning because new information may not be able to be acquired, stored or recalled.



Animal models provide insights into human memory and learning deficits following traumatic brain injury [36,40,105,106]. Animal learning and memory are often assessed using the radial arm or the Morris water maze [107-110]. Experimentally, anterograde or post-traumatic amnesia is considered a deficit in working memory (WM). Retrograde or pre-traumatic amnesia is classified as a reference memory (RM) deficit. WM deficits may be demonstrated if brain injury occurs prior to learning a maze task. RM deficits may be ascertained if rats are maze trained before receiving a brain lesion. Post-lesion performance will be affected by failure to accurately recall the correct responses.

Performance in the radial arm and water mazes involves spatial memory processing which is dependent on external landmarks or cues (allocentric spatial localization) [108-110]. This contrasts to egocentric spatial localization wherein internal cues or body orientation are used to solve a maze. For example, to solve a T-maze rats merely learn which way to turn (right or left). In rats severe deficits in allocentric spatial processing occur following lesions of the both parietal cortices or the right unilateral parietal cortex alone, ventrolateral orbital cortex or the hippocampus [111-118]. Egocentric spatial localization dysfunction is seen after unilateral and bilateral agranular medial cortex or caudate lesions [114,115,119]. Thus, rodent allo- and egocentric spatial localization appear to be mediated by different brain areas.

In humans and primates parietal cortex or hippocampal damage impairs allocentric spatial localization [120-124]. Humans with a parietal cortex lesion display deficits in comprehending external spatial arrangements or geography and have difficulty in forming an accurate external map of novel spatial environments [123]. Egocentric spatial processing deficits have been found in humans with frontal cortex damage and in monkeys with dorsolateral pre-frontal damage [121,123,124]. Patients with such lesions exhibit an impairment in right-left discrimination [124].

The right sensorimotor injury in our rat model also affects parietal and medial frontal cortices which are believed to be involved in memory and spatial localization functions. We thus tested the effects of this specific brain injury on both RM and WM by an eight arm radial maze. Two different baiting schemes allowed us to evaluate different search strategies used by the rats after injury.

MATERIALS AND METHODS

A) SUBJECTS

Fifty-four naive adult male Sprague Dawley rats, weighing 300-400 g. were used. They were housed in groups of four or five on a reverse light cycle (lights on 1100 to 2300 hours) and given free access to water. At the beginning of training they were on a restricted feeding schedule to maintain their body weights at 80-85% of free feeding levels, adjusted for growth.

MEMORY TEST

B) APPARATUS

The eight arm radial maze consisted of 8 identical arms (9 cm wide X 11 cm high X 71 cm long) radiating from an octagonal platform (34 cm wide). Food wells located 1.5 cm from the end of each arm were 2 cm in diameter. The wooden maze floor was painted black while the sides and the tops of both the alley-ways and the central platform were made of clear plexiglass. The maze was 82 cm above the floor; light at maze level measured 40 footcandles. Several distinct visual cues present in the room remained fixed throughout testing.

C) PRE-INJURY TRAINING--EXPERIMENT #1 (ALL 8 ARMS BAITED)

Rats (n=28) were tested once per day, five days a week. All rats were placed onto the central platform in the same manner and orientation. For the first two training days the rats became familiar with the apparatus as we scattered pieces of Kellogg's Froot Loops in the maze arms. Afterwards, formal training consisted of placing one half a Froot Loop in the food well of all 8 arms. Daily training continued until all 8 Froot Loops were obtained or 5 minutes had elapsed. Re-entry (i.e. all four paws placed into an arm) into any arm previously visited within any daily trial was scored as a working memory (WM) error. Training continued until a criterion of choice accuracy of at least 87.5% for the first 8 choices was attained for 5 consecutive days (i.e. at least 7 of the first 8 choices were correct). Training to criteria took 9.7 $\pm$ .8 days. Twelve animals used the strategy of going to each adjacent arm and were excluded from the study since this strategy may not depend upon allocentric spatial processing.

D) PRE-INJURY TRAINING--EXPERIMENT #2 (4 of 8 ARMS BAITED)

A separate group of rats (n=26) became familiar with the apparatus as in experiment #1. Afterwards training began but

only 4 of 8 arms were baited throughout the training and testing period. While the baiting scheme was consistent for any individual rat, it varied from rat to rat. An arm choice was recorded when a rat placed all four paws into the arm. Any re-entry into a previously visited arm was scored as a WM error while entry into never baited arms were scored as reference memory (RM) error. Any re-entry into an arm which was never baited was considered a combination of both a working and a reference memory error (WRM) and was scored separately. Sessions continued until all 4 Froot Loops were obtained while committing no errors for 4 out of 5 consecutive days. Training to this criterion took 45.5 ± 2.7 days.

E) STATISTICS

Experiment #1 (8 arms baited): The number of correct choices before each rat made the first WM error (i.e. correct entries to first error) as well as the total number of WM errors committed in obtaining all eight rewards were analyzed in blocks of three days by ANOVA for repeated measures.

To detect if there was a tendency to go to adjacent arms, the searching pattern of each rat was scored using the position of the arm chosen relative to the arm just visited. Going from arm #1 to arm #5 gives a score of 4, going from arm #2 to arm #3, gives a score of 1, while going from arm #6 to arm #4, yields a 2 and so on. The sum of these choice scores divided by the total number of choices made to obtain all eight Froot Loops yields a relative choice position "score". Each rat's relative choice position score for the seven days prior to and after brain injury was analyzed using ANOVA for repeated measures (pre-injury vs post-injury) with a planned contrast for the post-injury choice position score.

Experiment #2 (4 of 8 arms baited): Reference memory (RM) errors, WM errors and working/reference memory (WRM) errors (i.e. re-entry into never baited arms) were grouped into blocks of 3 days each and analyzed by ANOVA for repeated measures. Days to re-attain criteria performance after surgery and/or injury were compared by a t-test.

To determine if the rats displayed a directional bias in obtaining the reinforcements after brain injury we counted the number of right hand turns used by the rats in seeking rewards during the 4 errorless of the last five days when they initially attained criteria and again after brain injury when they re-attained criteria. The data were analyzed by ANOVA for repeated measures.

Results

The amount of cortical damage and axonal degeneration adjacent to the injury site two days after injury is depicted in Figure 20. Although there is a minimal amount of cortical loss, there is a penumbra area beneath and around the injury site undergoing axonal degeneration. By two weeks after injury there was a large increase in cortical cell loss resulting in a necrotic or gliotic cavity (Fig 21).

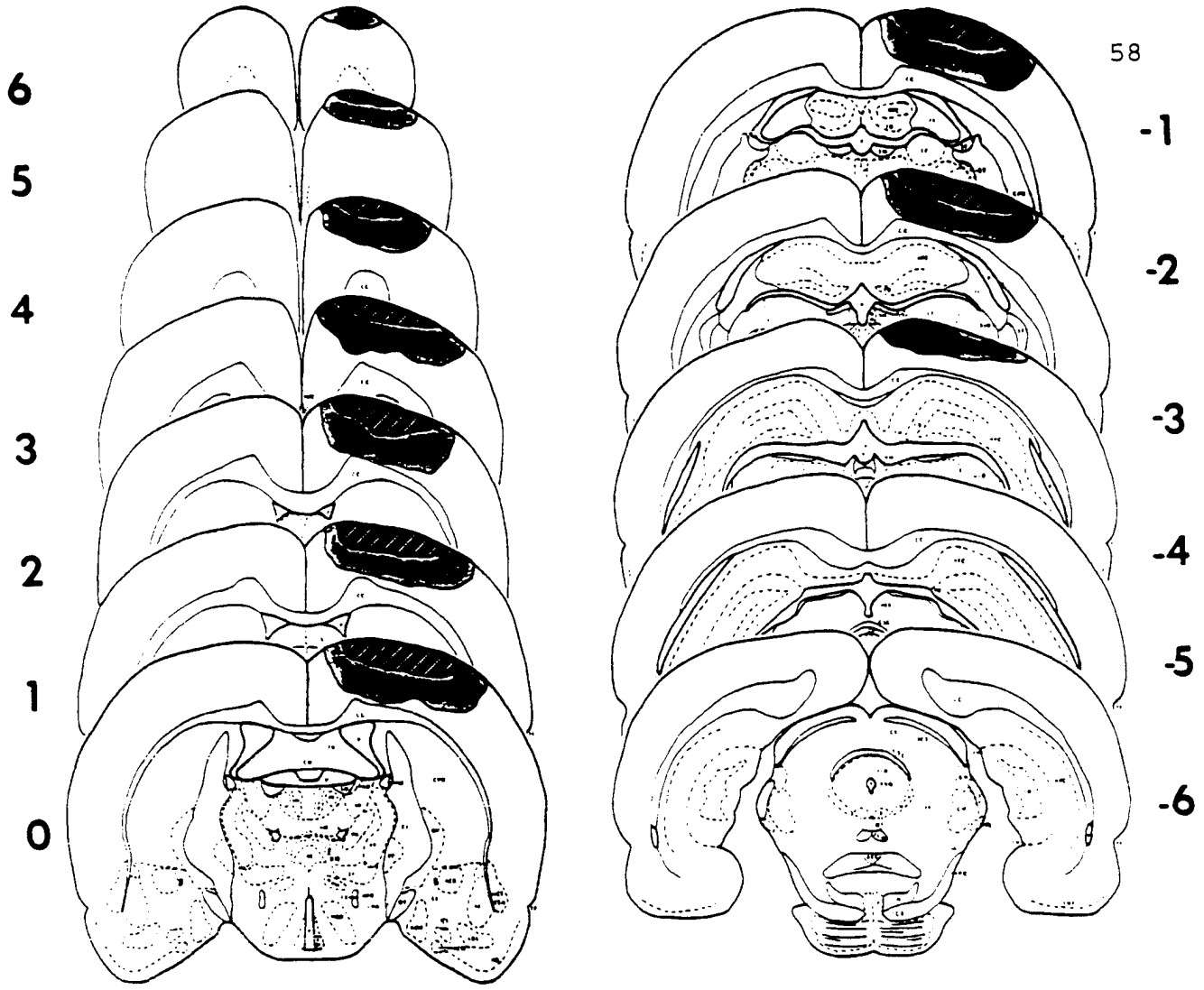


Figure 20. Cortical loss and axonal degeneration in the adjacent injury area two days after injury. Drawings taken from the Atlas of the rat brain [126]. Selected coronal sections at one millimeter intervals through the brain from stereotaxic coordinates +6.0 to -6.0 (AP). The dark cross-hatched area indicates the amount of cortical loss. The lighter shading indicates areas of detectable axonal degeneration. Each section represents the average of three brains. The parietal cortex extends approximately from 3mm to 6mm lateral of midline and from -2mm to -6mm (AP). The medial frontal cortex extends approximately from the midline to 2mm laterally and from +5 to -2mm (AP).

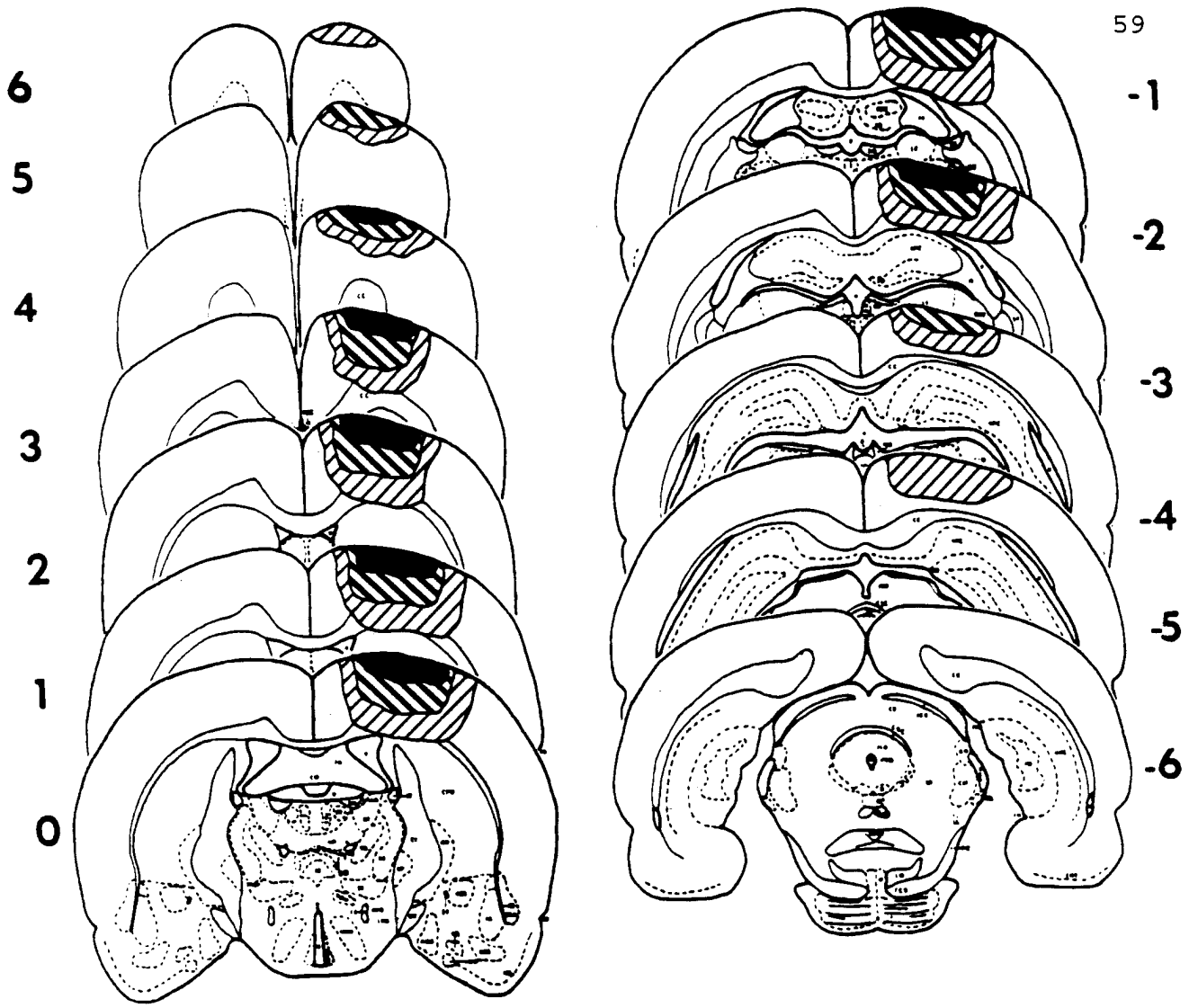


Figure 21. Cortical loss 2 weeks after brain injury. Drawings taken from the Atlas of the rat brain [126]. Selected coronal sections at one millimeter intervals through the brain from stereotaxic coordinates +6.0 to -6.0 (AP). The lightest shaded cross-hatched area indicates the maximal amount of neural parenchyma destroyed. The darkest cross-hatched area indicates the minimal amount of cortex destroyed and the intermediate cross-hatched area indicated by the thick cross-hatch lines illustrates the average amount destroyed by the impact injury. The parietal cortex extends approximately from 3mm to 6mm lateral of midline and from -2mm to -6mm (AP). The medial frontal cortex extends approximately from the midline to 2mm laterally and from +5 to -2mm (AP); (n=5).

B) EXPERIMENT #1---(ALL 8 ARMS BAITED)

1. Working Memory: ANOVA indicated that there was no main effect of injury nor an injury X time interaction indicating that right parietal brain injury did not cause WM errors. There was a main effect of time $F(4,104)=5.19$, $P < .001$ indicating that both injured and control rats performed better over time after brain injury, (data not shown)

2. Arm Entries Before the First Working Memory Error: ANOVA indicated that there was no main effect of injury or a time X injury interaction indicating that brain injury did not shorten the time until the first WM error was committed. There was a main effect of time $F(4,104)=2.46$, $P < 0.05$ indicating that both injured and control rats performed better over time, (data not shown)

3. Number of Arms Skipped Between Choices: ANOVA on the number of arms skipped between arm choices revealed a main effect of time (pre-injury vs post-injury), $F(1,26)=25.89$, $P < .001$ and a injury X time interaction, $F(1,26)=5.86$, $P < .03$. Further analysis using a planned contrast indicated that after injury the rats tended to enter adjacent arms sequentially more so than they did before injury $F(1,26)=10.51$, $P < .005$. Control rats did not shown this tendency (Table 1).

TABLE 1: Means and standard error of the mean (S.E.M.) for choice position scores in an 8 arm radial maze for 7 days before and 7 days after brain injury or surgical preparation without brain injury.

	CONTROL	INJURED
Pre	2.07 = .05	2.09 = .05
Post	1.86 = .05	1.49 = .06*

* indicates significantly different from pre-injury value. $P < .005$

Inspection of the response characteristics of individual rats showed that nine of 14 injured rats went to 7 or 8 arms in sequence (3 rats for 7 days, 3 rats for 6 days and 3 rats for 4 days post-brain injury) and two rats for one or two days. Three injured rats never displayed sequential arm entry.

B) EXPERIMENT #2---(4 OF 8 ARMS BAITED)

1) Reference Memory: Brain injured rats made more errors than controls, $F(1,24)=29.4$, $P<.0001$. There was a main effect of time, $F(4,96)=38.87$, $P<.0001$ and a time X injury interaction, $F(4,96)=6.34$, $P<.0001$ indicating that the effects of brain injury on RM differed at specific points after injury when compared to the control group. The RM impairment persisted for 12 days after injury ($P<.05$, Tukey's test) (Figure 22).

2. Working Memory: Brain injured rats did not commit more WM errors than controls $F(1,24)=2.04$, $P<.17$. There was a main effect of time $F(4,96)=8.52$, $P<.0001$ but there was no interaction indicating that the WM errors decreased over time similarly in both the injured and control groups (Figure 22).

3. Working/Reference Memory: ANOVA indicated that brain injured rats re-entered previously un-baited arms more so than controls (WRM errors) $F(1,24)=7.08$, $P<.014$. There was a main effect of time $F(4,96)$, $P<.01$ indicating the number of WRM errors decreased for both groups of rats. Lack of a time X injury interaction indicates that the injured animals committed more WRM errors than controls but that the errors were not associated with a particular time point (data not shown).

4. Time to Re-attain Criterion: Brain injured rats took longer to re-attain criteria (19.2 + 1.6 days) than control rats (10.8 ± 1 days) $t=4.36$, $P<.0001$ (Figure 23), but took less time than it took to learn the maze when they were naive (19 vs 45 days).

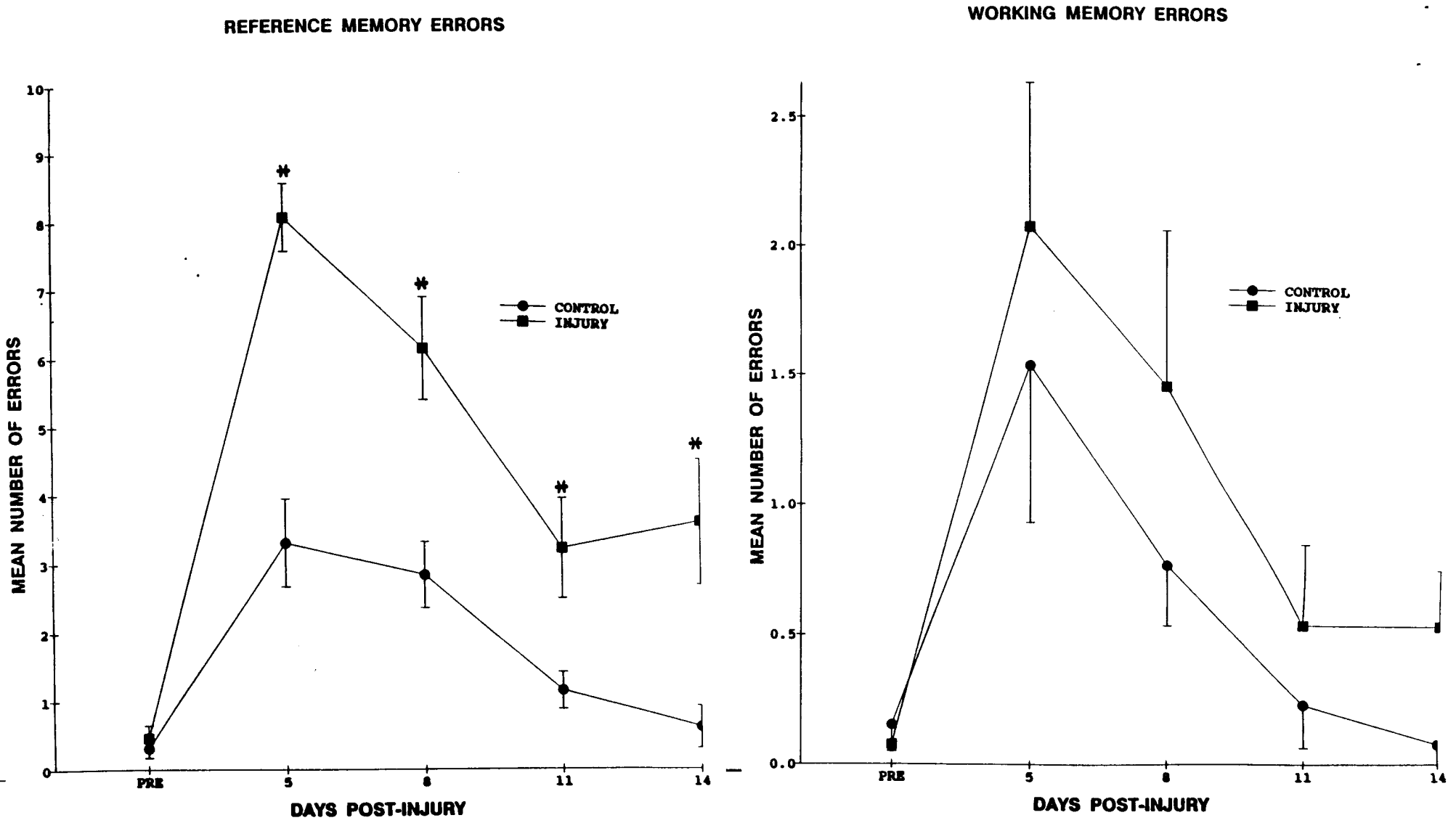


Figure 22. Rats were trained to criteria with 4 of 8 arms baited prior to TBI. Testing began 2 days post-TBI. Each data point represents a 3 day block. n=13 per group. * = P<.05 vs control, Tukey's test.

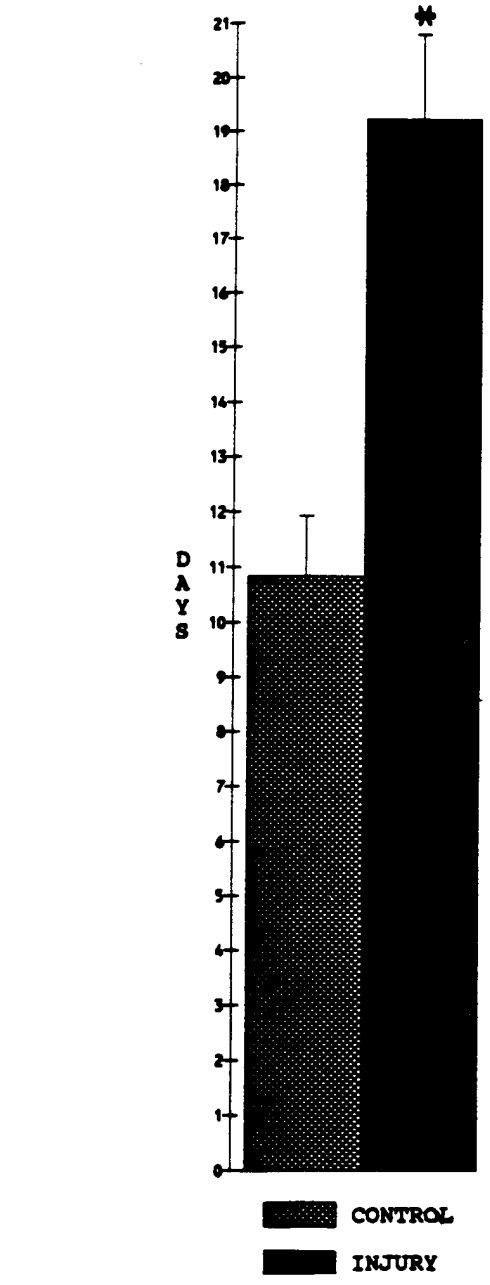


Figure 23. Rats were trained to criteria with 4 of 8 arms baited prior to either brain injury or sham surgery (control). Testing began 2 days post-TBI. Criteria = no RM, WM or WRM errors for 4 out of 5 consecutive days. n=13 per group. * = P<.0001; T-test.

5. Directional bias: ANOVA indicated that after brain injury there was a significant group X time interaction, $F(1,24)=15.78$, $P<.0006$. The planned contrast indicated that after brain injury rats had a directional bias in re-learning which 4 arms were baited $F(1,24)=31.56$, $P<.0001$ (Table 2)

TABLE 2: Means and standard error of the mean (S.E.M.) for the number of right hand turns in performing the 8-arm radial maze when only 4 arms were baited.

	CONTROL	INJURED
Pre	1.69 = .16	1.27 = .15
Post	1.69 = .16	2.94 = .08*

* indicates significantly different from pre-injury value
P <.0001

Discussion of Memory Deficits Following Traumatic Brain Injury

Our model of brain injury which affected the entire right sensorimotor cortex did not affect working memory (WM) but did cause three separate, but related, effects: 1) a temporary deficit in reference memory; 2) a deficit in allocentric spatial localization with an apparent shift to egocentric spatial localization; 3) a distinct right hand bias.

Reference Memory (RM): This deficit was detected when only 4 of 8 arms were baited. Injured rats displayed a significant deficit in RM when they entered arms which they previously learned were un-baited (experiment #2; Fig. 22). This RM deficit was temporary. Over time the injured rats fully recovered their RM but took almost twice as long to re-learn the maze and re-attain response criteria than the control rats (19 days vs 10 days; Fig. 23). This 19 day re-learning interval was less than half the 45 days it took to originally attain criteria before injury indicating that the rats did not revert to a "maze naive" learning set after injury but retained some knowledge about the maze baiting scheme. Learning or acquisition functions were not permanently impaired. Our rats showed no hippocampal cell changes (cresyl violet or hematoxylin & eosin) from 2 days to 8 weeks after injury (unpublished data).

Other investigators have produced RM deficits in rats by a lateral parietal fluid percussion impact, but in these experiments unilateral or bilateral dentate hilar neuron loss occurred [105, 106].

Working Memory (WM): Injury did not cause a WM deficit in our rats because injured rats did not re-enter previously baited maze arms more so than controls. Lack of a WM deficit is consistent with the lack of hippocampal histologic changes in our model because an increase in WM errors is usually associated with hippocampal cell loss [110].

Some investigators however have produced WM deficits with midline fluid percussion or cortical impacts which did not produce light microscopic evidence of either cortical or hippocampal damage. These rats did not exhibit RM deficits [31, 40].

Rats have only slight RM impairments even after extensive bilateral hippocampal lesions indicating that the information necessary to perform spatial tasks must be stored in non-hippocampal brain areas [127, 128]. Bilateral parietal cortex ablations in rats produce RM deficits [129]. Bilateral medial prefrontal cortex ablations cause a temporary RM deficit but

these ablations are associated with a long lasting WM deficit [129].

The major injury in our rats involved the right sensorimotor cortex, most of the right parietal cortex and, inconsistently, some of the ipsilateral medial frontal cortex. Since bilateral medial prefrontal cortical lesions are associated with WM deficits, which our rats did not display, the variable unilateral medial frontal damage in our rats was insufficient to produce this deficit but it could have contributed to their RM deficit. The major cortical injury in our rats involved the parietal lobe which supports the contention that the parietal cortex may play a key role in RM and further that a right parietal cortex lesion alone is sufficient to cause a RM deficit.

Working/Reference Memory (WRM): Unexpectedly, we found that after injury the animals more often made re-entries into never baited arms than did controls ($P < .014$, see Results; 4 of 8 arms baited). This is considered a working memory error committed on the reference memory components of the radial maze and is classified as a WRM error. To our knowledge finding significant WRM errors, but not WM errors, has never before been reported. When we considered WRM errors as purely WM errors and re-analyzed the data by ANOVA for repeated measures, there was again no significant effect of injury on WM ($P < .09$). We are unable to offer a good explanation at this time other than to suggest that since the WRM errors represent a deficit in both RM and WM this finding may be more the result of RM than WM.

Search Strategies: A shift in response strategy and a uniform directional bias toward the injured side (right) occurred after right parietal brain injury. This effect was seen whether all arms or just 4 of 8 arms were baited.

Before injury the rats did not obtain the Froot Loops by going to adjacent arms even though all eight arms were baited. After injury the rats immediately began searching for the Froot Loops by going to adjacent arms. This stereotypic response continued for at least 7 days. Afterwards, five rats resumed pre-injury searching patterns while nine rats retained the stereotypic response pattern. Though the search strategies used by the injured and control rats differed (stereotypic vs non-stereotypic), there was no statistical difference in the number of WM errors committed between the two groups. In all cases the stereotypic search strategy included movement toward the side of the brain injury, while the controls showed no directional bias.

The rats which were trained to go to only 4 baited arms also showed a right hand directional bias and a propensity to go to

adjacent arms for from 2 to 6 days after injury. After using this stereotypic strategy, the animals then re-learned which 4 arms were baited and successfully re-attained criteria. We hypothesize they used yet another search strategy probably based on egocentric cues.

Rats are believed to solve the radial arm maze by allocentric spatial localization controlled by the right parietal cortex [115]. The medial frontal cortex governs egocentric spatial functions [114,115]. Egocentric spatial localization does not appear to be lateralized because either right or left medial frontal cortex lesions cause mild and equal amounts of egocentric spatial localization deficits. Bilateral lesions cause cause significantly more deficits than does a unilateral lesion [115].

Since the right parietal cortex, necessary for allocentric spatial localization, was severely injured in our rats and the right medial frontal cortex only slightly so, the neural substrate necessary to support egocentric localization remained (medial frontal cortices). The switch to an egocentric strategy was an adaptive mechanism manifest by the stereotypic behavioral response pattern of entering adjacent arms. This strategy required only that the rat make a sharp right (or left) hand turn after exiting each arm. A similar switch from a non-stereotypic strategy to a stereotypic strategy has been reported in rats after medial septal lesions [130].

The egocentric stereotypic search strategy was adaptive when all eight arms contained a Froot Loop but was not so when only 4 of 8 arms were baited (experiment #2). Thus the brain injured rats eventually abandoned the egocentric adjacent arm entry strategy and correctly entered baited arms without going from arm to arm. Whether they returned to an allocentric spatial localization search strategy or used an egocentric strategy variant is uncertain. A return to an allocentric strategy may have been possible because of takeover by the remaining parietal cortex (the entire parietal cortex was not damaged in our rats) or perhaps by takeover by other cortical areas, even on the left side of the brain [112]. An alternative egocentric strategy could also have been used. The rats could have re-learned the maze by learning a response pattern based on turns and angles to the next baited arm rather than using extramaze cues (i.e response chaining). Since the injured rats had a right hand bias, it is easy to conceive that as long as the rat could reliably locate the same initial arm upon being put into the maze, it could learn the location of the other three arms by learning the angle to take to the next arm upon exiting the arm it just was in. This appears to be exactly the strategy used by

8 of 13 rats because they re-attained criteria performance by going to the same initial arm and then the remaining 3 baited arms in the exact order. Before injury this strategy was not evident in our rats. The other 5 rats showed variable initial arm selections and arm entry patterns and thus may have relearned the maze using allocentric localization.

The most severe RM and allocentric localization deficits were early after injury when cortical loss was small (fig 20). Over time cortical loss, axonal degeneration and presumably cell loss increased at the injury site (fig 21), yet the rats regained RM and allocetric spatial localization or developed adaptive strategies i.e. egocentric spatial localization. This implies that an initial small amount of cortical damage starts neural reorganization in brain areas other than that which was injured.

Right Hand Turning Bias: The right hand bias displayed after brain injury by most of the rats may have several explanations: First, the rats may have been exhibiting contralateral neglect which results from unilateral lesions of the parietal or medial frontal cortex [118,131-134]. Crowne et al. [132] also found that parietal cortex lesions caused contraversive turning while medial frontal cortex lesions caused ipsiversive turning. Our injured rats had an ipsiversive (right hand) bias, suggesting that the bias may be the result of the minor damage to the medial frontal cortex despite significant parietal cortex damage. Prior motor analyses in rats similarly injured did not indicate an overt tendency to circle although they had a distinct right forepaw bias [135]. We did not specifically test for contralateral neglect nor systematically study circling behavior, but we have never observed overt circling behavior in our injured rats [135].

Second, the right side sensorimotor cortex lesion and/or medial frontal cortex damage may also have induced a striatal dopamine asymmetry. Striatal dopamine asymmetries are believe to be causally linked to motor asymmetries since higher dopamine levels are found in the striatum contralateral to an animal's preferred turning direction [136]. Indeed it has been shown that a rat's preference to turn left or right in a T-maze or which paw it chooses in an operant lever paradigm is correlated with its naturally occurring dopamine asymmetry in the striatum [137]. Behavior may even modify striatal dopamine concentrations asymmetries [138,139]. Since the right extremities of our brain injured rats were not paretic, they were preferentially used in the naturally occurring motor movements of rearing, landing and support. This continued use of the right extremities may have induced a dopamine asymmetry in various components of the motor system which caused the observed right hand turning bias.

Third the ipsilateral bias may have resulted from indirect damage via transsynaptic degeneration throughout the ipsilateral motor system. Striatal and substantia nigra lesions are well known to induce ipsiversive turning [140]. Lesions to the forepaw sensorimotor area have been shown to induce cellular degeneration in the ipsilateral caudate nucleus, substantia nigra and thalamic nuclei concerned with motor function [141]. We have recently described a pattern of axonal degeneration associated with our injury model which strongly suggests transsynaptic degeneration occurs into the cerebellum. Possibly after injury transsynaptic degeneration occurs throughout the entire motor system as a consequence of sensorimotor cortical injury [135].

Fourth, the frontal cortex, which is partially injured in our rats, is believed to have a moderating influence on nigro-striatal function. Unilateral frontal cortex lesions cause amphetamine-induced ipsiversive circling up to 7 days [142].

In summary, a right side sensorimotor/parietal injury results in a temporary RM, but not WM, deficit. Normal allocentric spatial localization functions are impaired and are replaced with egocentric localization strategies. A right side (ipsiversive) motor response bias also occurs which may influence the expression of the egocentric search strategy. Thus when injury or disease affects brain area(s) mediating a primary strategy, alternate strategies mediated by unaffected area(s) may be used. These secondary, alternate strategies may in turn be supplanted by additional strategies as the brain injured animal seeks behavioral efficiency.

4. Neurochemical Studies (Drs. Homayoun, Rodriguez deTurco and Bazan)

Background

After traumatic brain injury secondary, delayed chemical events occur which contribute to irreversible brain damage. These events are believed to be initiated by the release of neurotransmitters leading to the activation of their receptors. Receptor activation subsequently causes an increase in intracellular calcium and the activation of phospholipse A_2 (PLA_2) and phospholipase C (PLC). Activation of phospholipases and calcium-mediated secondary injury processes result in the release of free fatty acids (FFA) and diacylglycerol (DAG). FFA can contribute to secondary brain damage by direct mechanisms (i.e. uncoupling of mitochondrial function) or indirect mechanisms (i.e. 20:4 conversion to prostaglandins and leukotriens).

The metabolism of specific pools of phospholipids in excitable membranes is acutely sensitive to cerebral ischemia and trauma [143] Brain membrane phospholipids are highly enriched in polyunsaturated fatty acids (PUFA), especially arachidonate (20:4n-6) and docosahexaenoate (22:6n-3) [144-146]. Release of free arachidonic acid is the rate-limiting step in the cascade that results in the formation of biologically active eicosanoids. Furthermore, PLA_2 also leads to the synthesis of platelet-activating factor (PAF) [147-150], and alkyl-ether phospholipid which is a potent mediator of inflammatory and immune response proccesses [150].

In this report we present changes which occur in FFA and DAG in different brain areas following piston impact on the right sensorimotor cortex.

Methods

Anesthetized rats were injured on the right forelimb/hindlimb sensorimotor area and sacrificed at either 30 mins, 1,4, or 35 days after injury. Rats sacrificed 30 minutes after injury were continually anesthetized and decapitated and their brains rapidly fixed in liquid nitrogen. Rats sacrificed at 1,4, or 35 days were anesthetized with ether and then subject to cranial or microwave irradiation. For rats sacrificed at 30 minutes only the right and left hemispheres were sampled. For all other rats the brain was removed and dissected into 8 areas: right and left upper cortex, right and left lower cortex, right and left hippocampus, cerebellum, and brain stem. The brain

areas were immediately homogenized in chloroform/methanol (2:1 v/v). Lipids were then extracted [151] from these fractions and the protein [152] and phosphorus [153] content measured.

Free fatty acids and DAG were separated by thin-layer chromatography and derivatized to fatty acid methyl esters for quantification by gas-liquid chromatography [154].

Results

Table 3, page 73, demonstrates changes in FFA obtained from right (injured) and left (contralateral) hemispheres 30 mins after injury. In the injured hemisphere a significant increase in the release of C18:1, C20:4 and C22:6 (oleic, arachidonic and docosahexaenoic acid) was observed as compared to sham controls. No significant change was observed in left (contralateral) hemisphere.

Owing to high individual variations between animals in the other experimental groups, (1 day, 4 days and 35 days after injury) results for these groups are shown as the average values for the control animals (3 in each group) and **individual** values for experimental rats at different time points after trauma.

Total FFA content of different brain areas 30 mins, 1,4 or 35 days after injury is shown in figures 24A and 24B. Four days after injury a major increase in total FFA occurred in the area surrounding the injury (upper right cortex) and in the corresponding cortex in the contralateral hemisphere (upper left cortex), figure 24A. In the lower right cortex a high accumulation of FFA was observed 1 day post trauma but the FFA levels decreased thereafter.

Total FFA changes in the right hippocampus were inconsistent. The increase in total FFA in the left hippocampus was much higher 1 day after injury than at 4 or 35 days. No appreciable change was observed in total FFA of cerebellum (Fig. 24B). In brain stem, total FFA content showed the same pattern as in left hippocampus: it was increased 1 day after injury and decreased thereafter.

Results of **individual FFAs** in different brain areas for the different period after trauma are shown in figures 25, 26,27 and 28. One day after injury the major change in upper right and left cortices was in the level of 18:0 (Fig. 25). All FFAs were increased in the lower cortex without significant accumulation in lower left cortex. Although in right hippocampus only one rat showed high levels of all FFAs, in left hippocampus the increase in individual FFA was more accentuated.

Four days after injury, the increase in all FFAs in upper right and left and lower left cortices was more significant than at 1 day (Fig. 26). All FFAs were increased in both right and left hippocampus.

Compared to the other time points, individual FFA levels 35 days after injury were much lower in all areas of the brain and similar to sham animals. No major change was observed in the content of individual FFAs in cerebellum (Fig. 28) at different times after trauma. In brain stem, 18:0 and 18:1 were the fatty acids that showed higher accumulation.

The total diacylglycerol content (DAG-acyl group) extracted from different brain areas is shown in figures 29A and 29B. All injured rats had an increase in total diacylglycerol (DAG) content in brain surrounding the injury from 1 to 35 days following the injury. In the contralateral cortex (upper left cortex) the increase in DAG was more accentuated at 4 days and remained significantly elevated at 35 days after the trauma. In lower areas of cortex a similar pattern of increase in DAG-acyl group was observed for both right and left sides at 1 day and 35 days post-trauma. In the hippocampus, no major change was observed regardless of post-traumatic time.

Individual fatty acyl content of DAG are shown in figures 30,31,32 and 33. One day after injury in upper right and left cortex, the major change is in the levl of 18:0- and 20:4- DAG. The same increase was observed in the lower right cortex. 20:4-DAG increased in both hippocampi. Four days after trauma, while in upper left cortex the levels of all DAG-acyl groups remained high, in upper right cortex only a higher level of 18:0-DAG was observed. In lower right and left cortex and left hippocampus, all DAG-acyl groups showed lower levels compared to the controls. At 35 days post-trauma, in right and left cortex and both upper and lower portions, significantly higher levels of all fatty acids were observed. However, the levels of all fatty acids were lower compared to corresponding sham values. Both the cerebellum and brain stem showed higher levels of all DAG-acyl groups at 1 and 35 days after traumatic injury.

Changes in total lipid phosphorus content of all brain areas are shown in Fig 34. A decrease in total lipid phosphorous was observed only in right upper cortex and left hippocampus at day 1. A decrease in total lipid phosphorous was then noted in lower cortical areas by day 4 and eventually in the left hippocampus and cerebellum by day 35.

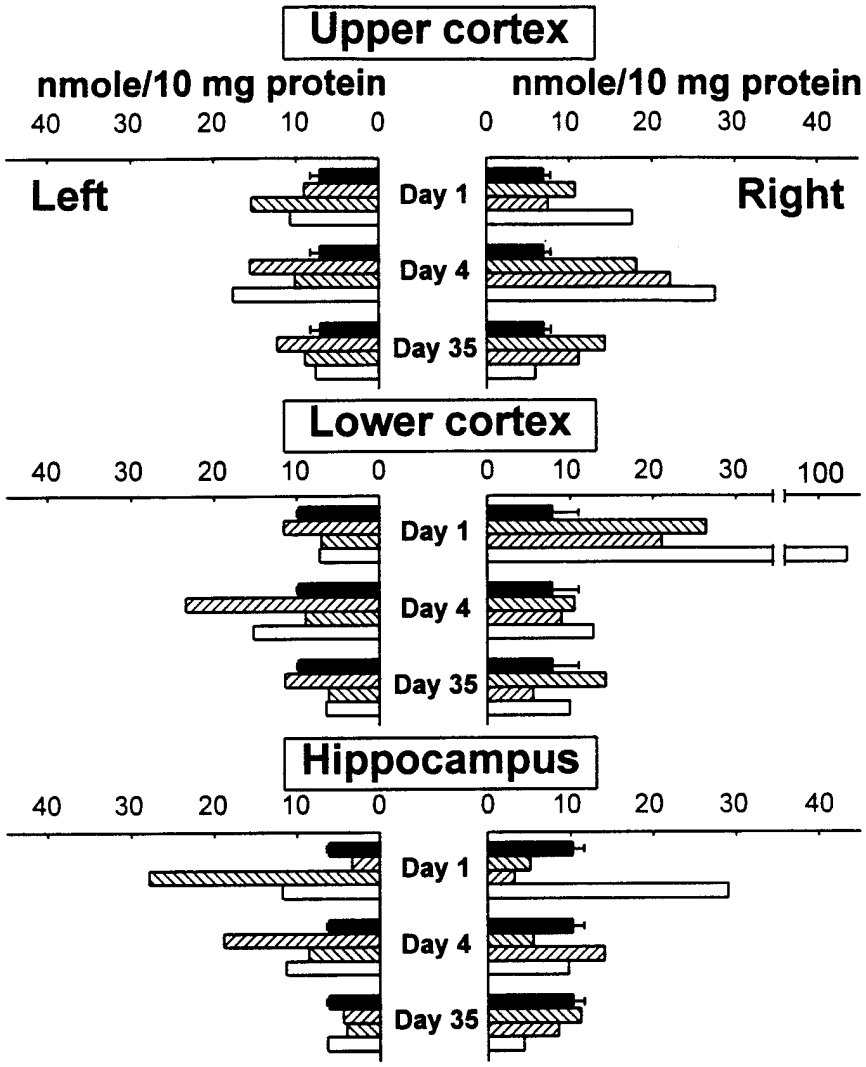
Table 3

Changes in free fatty acid content of cerebral hemisphere in sham operated controls and experimental rats 30 minutes after traumatic brain injury

Fatty acid	Right hemisphere		Left hemisphere	
	Sham	Exp	Sham	Exp
16:0	5.0±2.1	7.9±1.9	4.4±1.3	4.0±1.6
18:0	11.8±3.4	15.8±3.4	9.3±1.5	5.1±1.8
18:1	1.0±0.5	2.6±0.2*	2.8±0.4	1.8±0.9
20:4	ND	3.1±0.4*	0.5±0.2	1.3±1.0
22:6	0.2±0.1	0.5±0.1*	0.4±0.2	0.4±0.3
Total	19.2±5.7	30.5±6.1	20.8±1.8	16.4±9.1

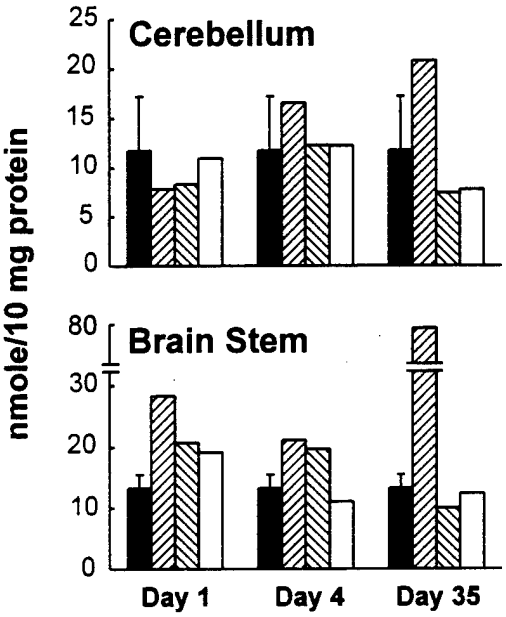
Values are expressed in nmole/10mg protein and are the mean±SD of 2-5 animals. ND= not detected.

Figure 24A: Total free fatty acids in the rat cerebral cortex and hippocampus after traumatic injury



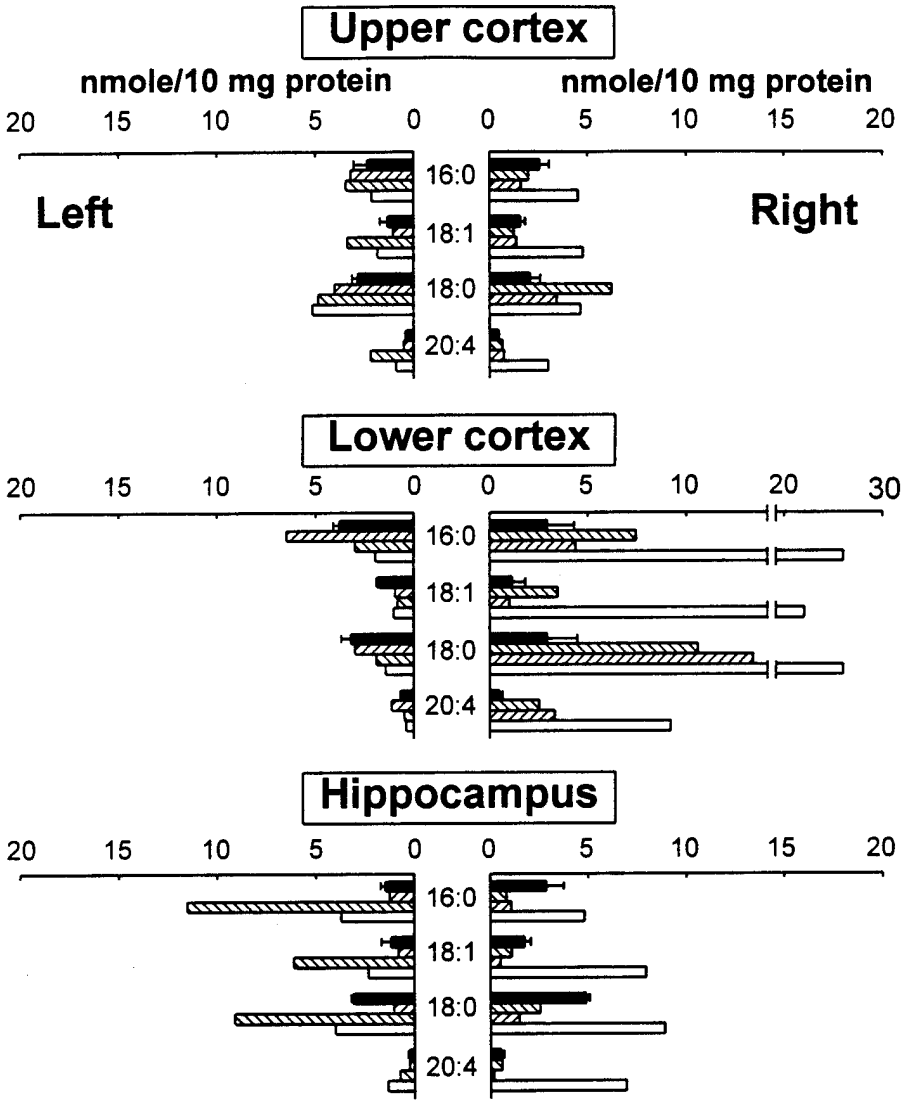
Total free fatty acids in the cerebral cortex and hippocampus of injured (right) as compared to contralateral (left) hemisphere as a function of time post-trauma. Mean sham control values ± SD (■, n=3) are compared to experimental rats (▨, ▧, □, represent one rat each) at different day post-trauma.

Figure 24B: Total free fatty acids in the rat cerebellum and brain stem after traumatic injury



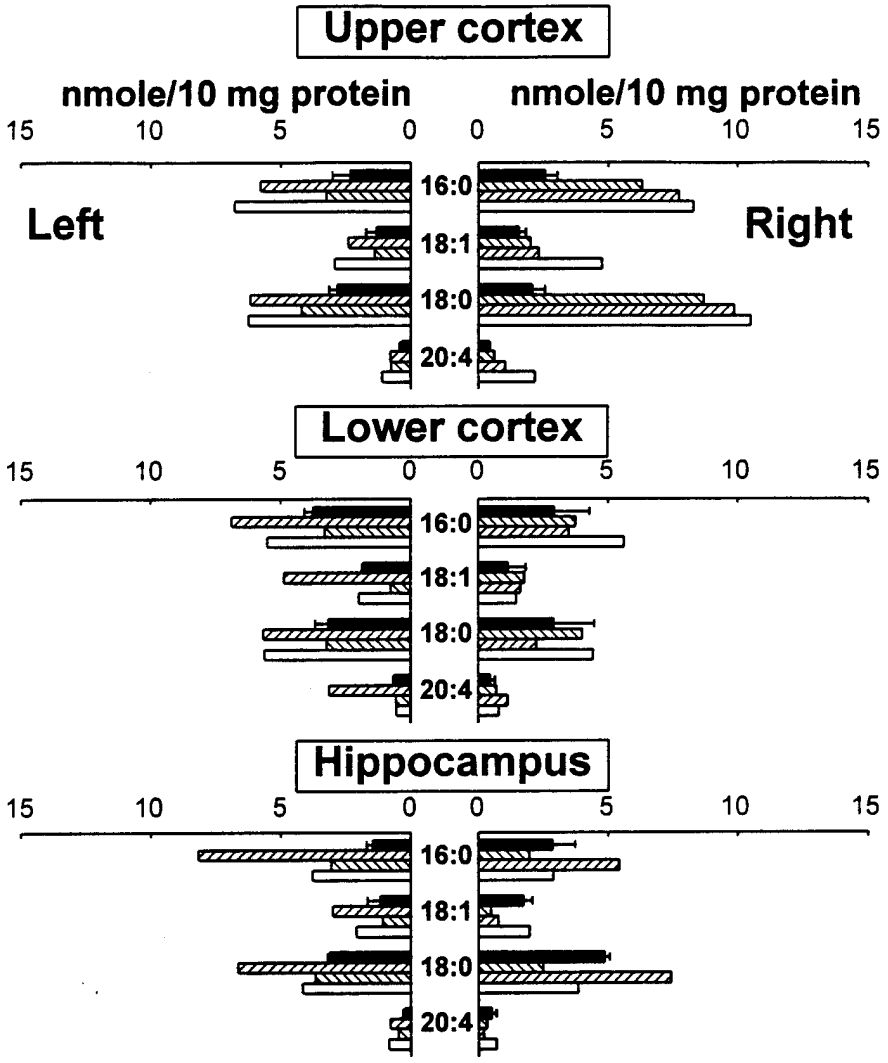
Bars represent total free fatty acids/10 mg of protein in the cerebellum and brain stem at 1, 4, and 35 days post-trauma. Sham control values (■, n=3) are compared to experimentalanimals (▨ , ▧ ,□ , represent one rat each) for each experimental group

Figure 25: Free fatty acids in cerebral cortex and hippocampus at day 1 post-trauma



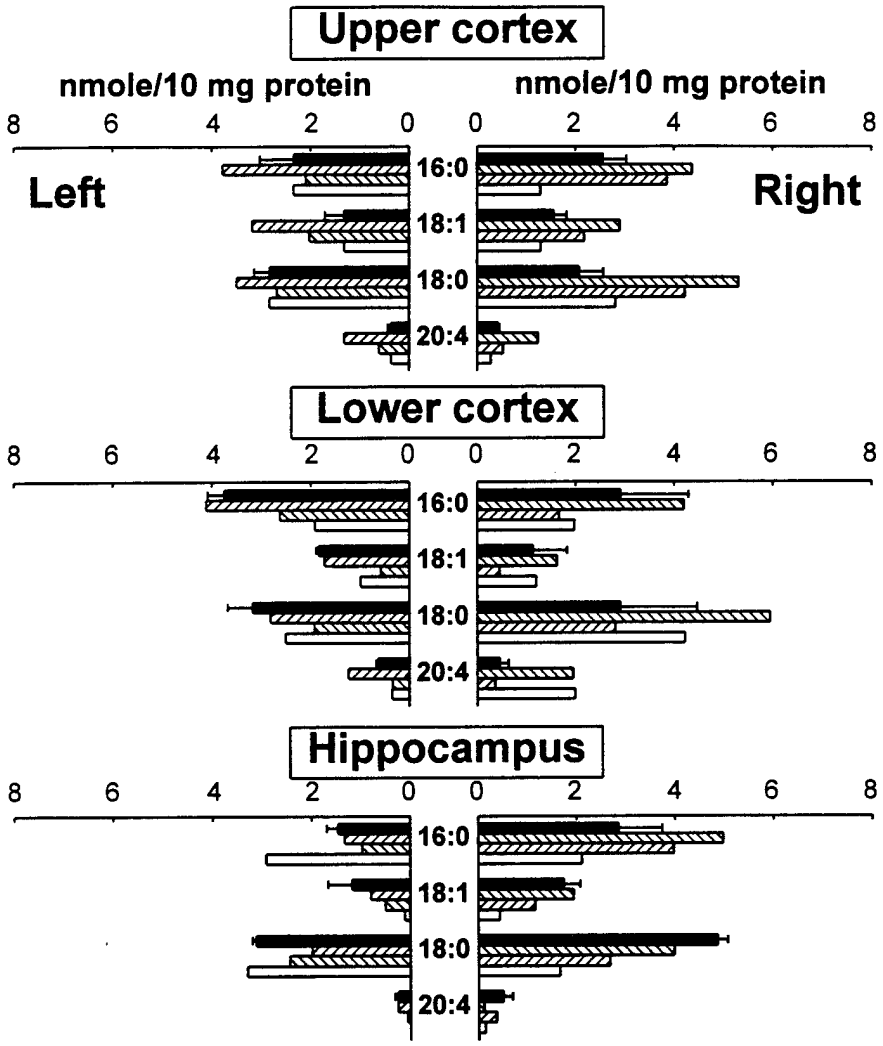
Level of individual free fatty acids in the cerebral cortex and hippocampus from injured (right) as compared to contralateral (left) hemisphere. Sham control values (■, n=3) are compared to experimental animals (▨, ▧, □, represent one rat each).

Figure 26: Free fatty acids in cerebral cortex and hippocampus at day 4 post-trauma



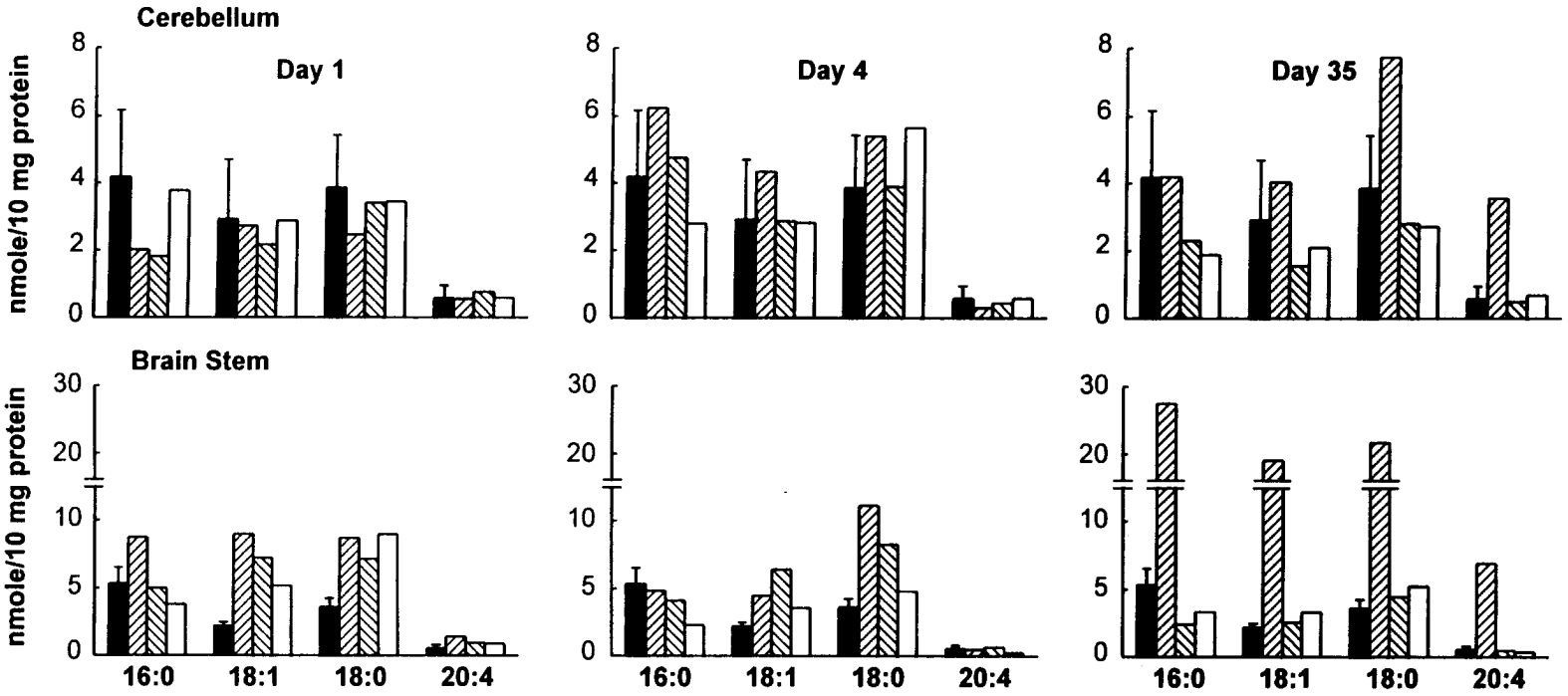
Details as in Figure 25

Figure 27: Free fatty acids in cerebral cortex and hippocampus at day 35 post-trauma



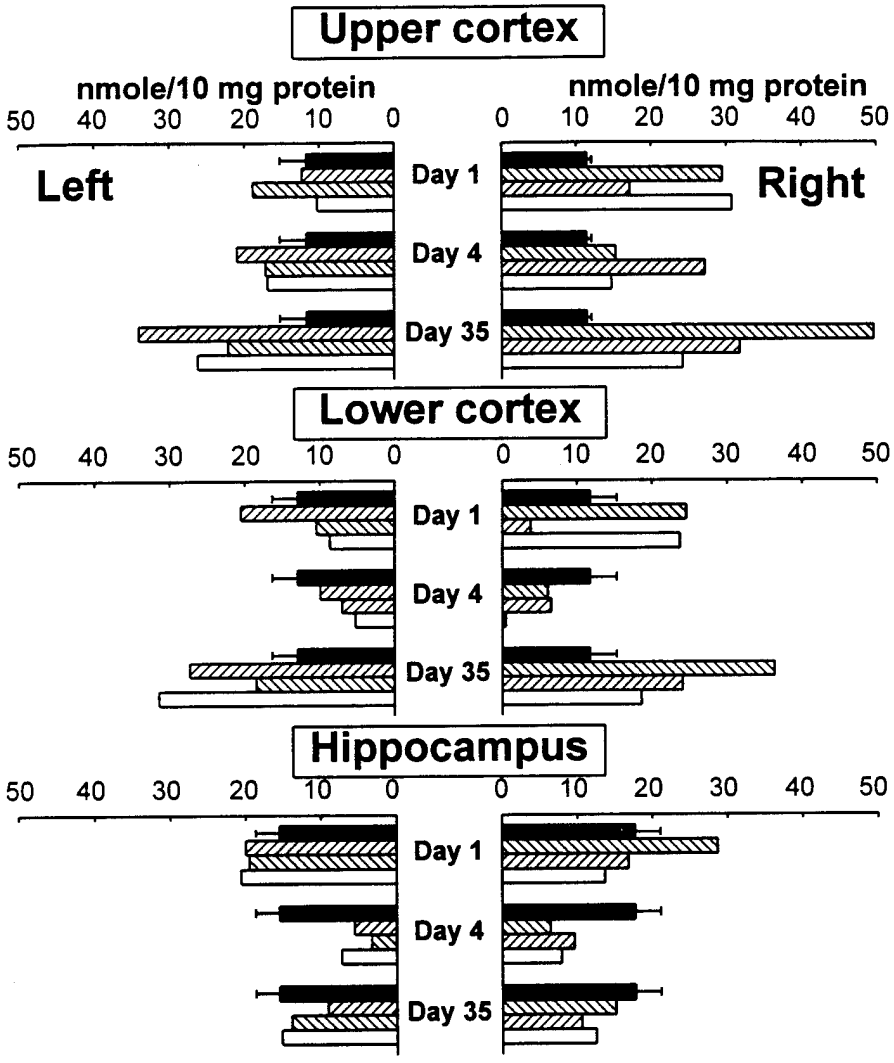
For details see Figure 25 legend.

Figure 28: Free fatty acids in the cerebellum and brain stem of rats after traumatic injury



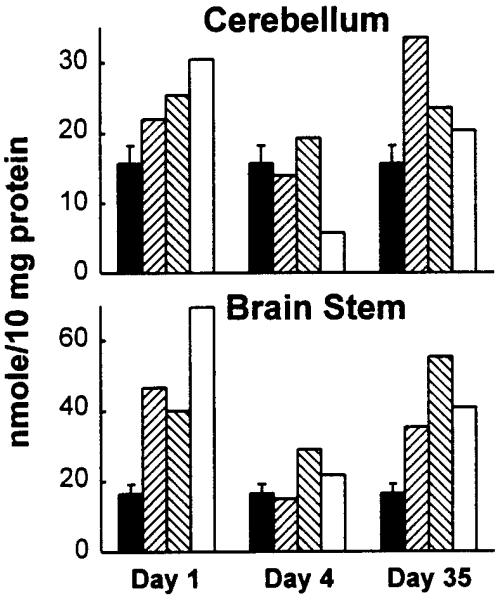
For details see Figure 25 legend.

Figure 29A: Total diacylglycerol in the rat cerebral cortex and hippocampus after traumatic injury



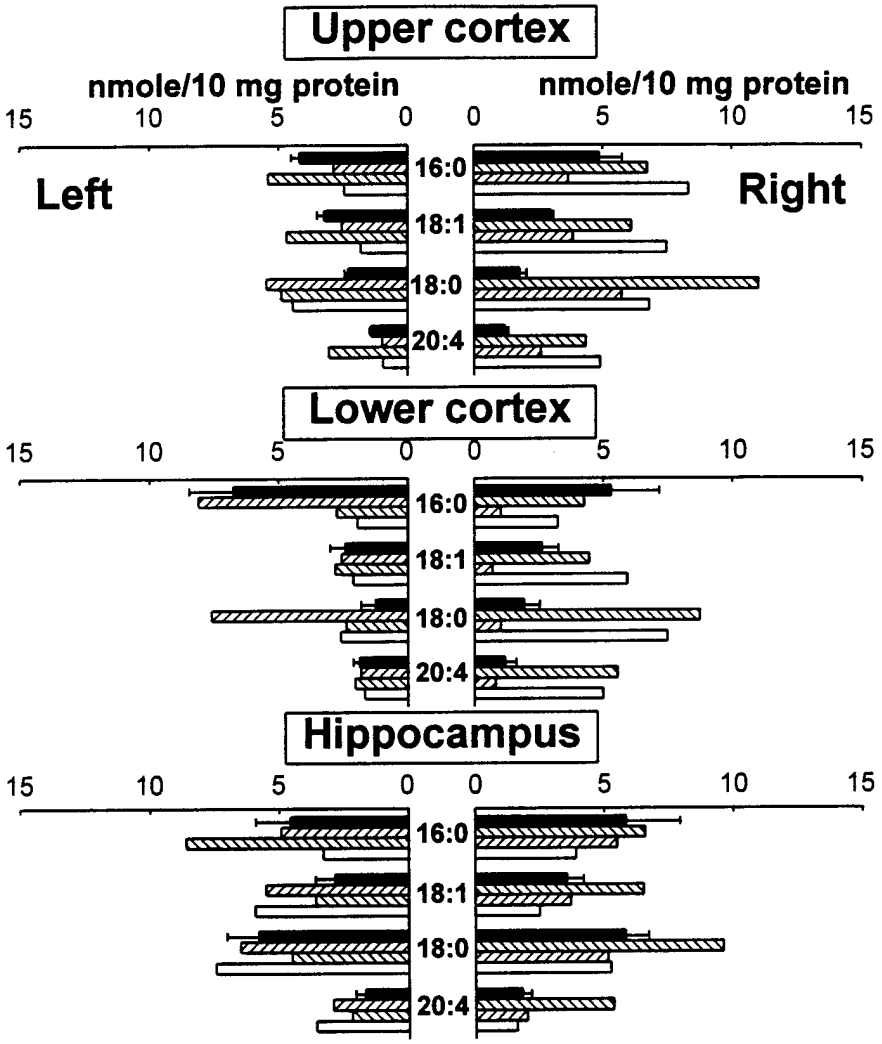
Total diacylglycerol- acyl groups in cerebral cortex and hippocampus from injured (right) and contralateral (left) hemisphere. Sham control values (■, n=3) are compared to experimental animals (▨, ▧, □, represent one rat each at different days post-trauma.

Figure 29B: Total diacylglycerol in the rat cerebellum and brain stem after traumatic injury



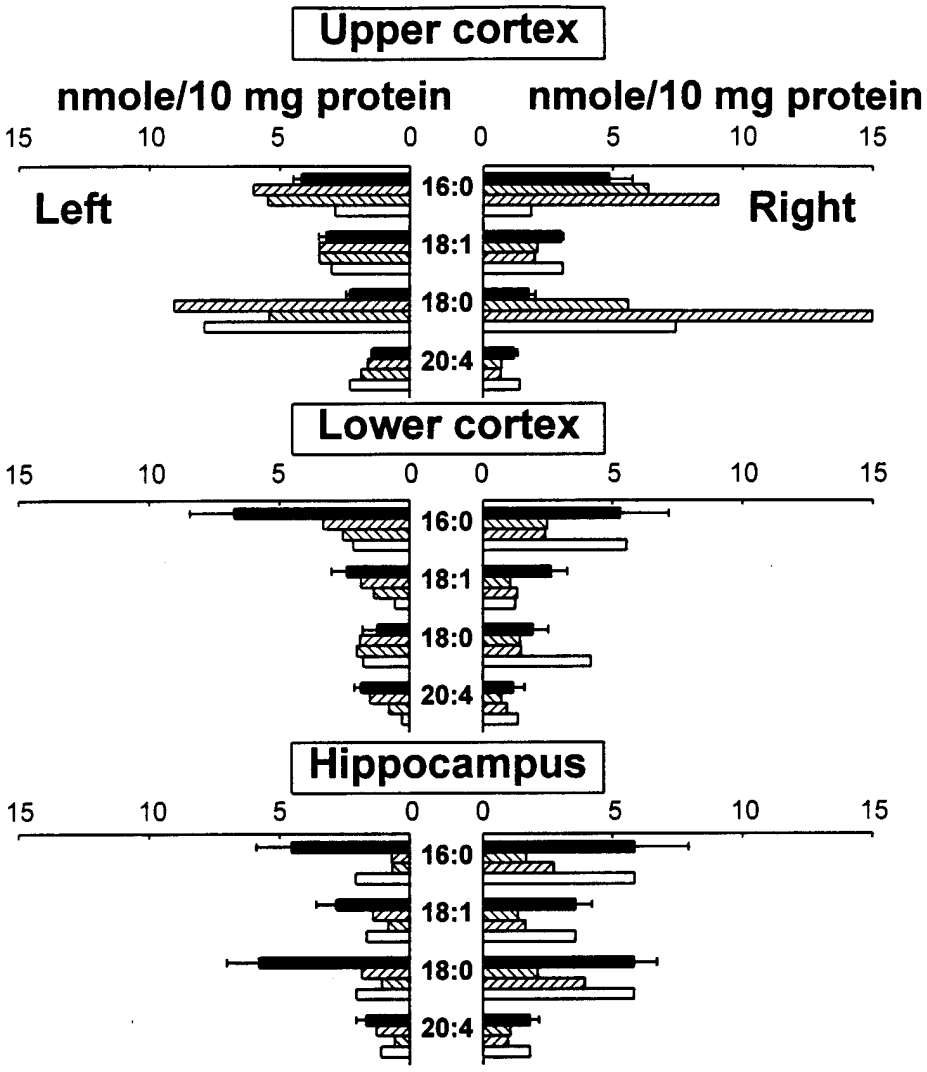
Details as in Figure 24 B legend.

Figure 30: Diacylglycerol acyl groups in the rat cerebral cortex and hippocampus at day 1 post-trauma



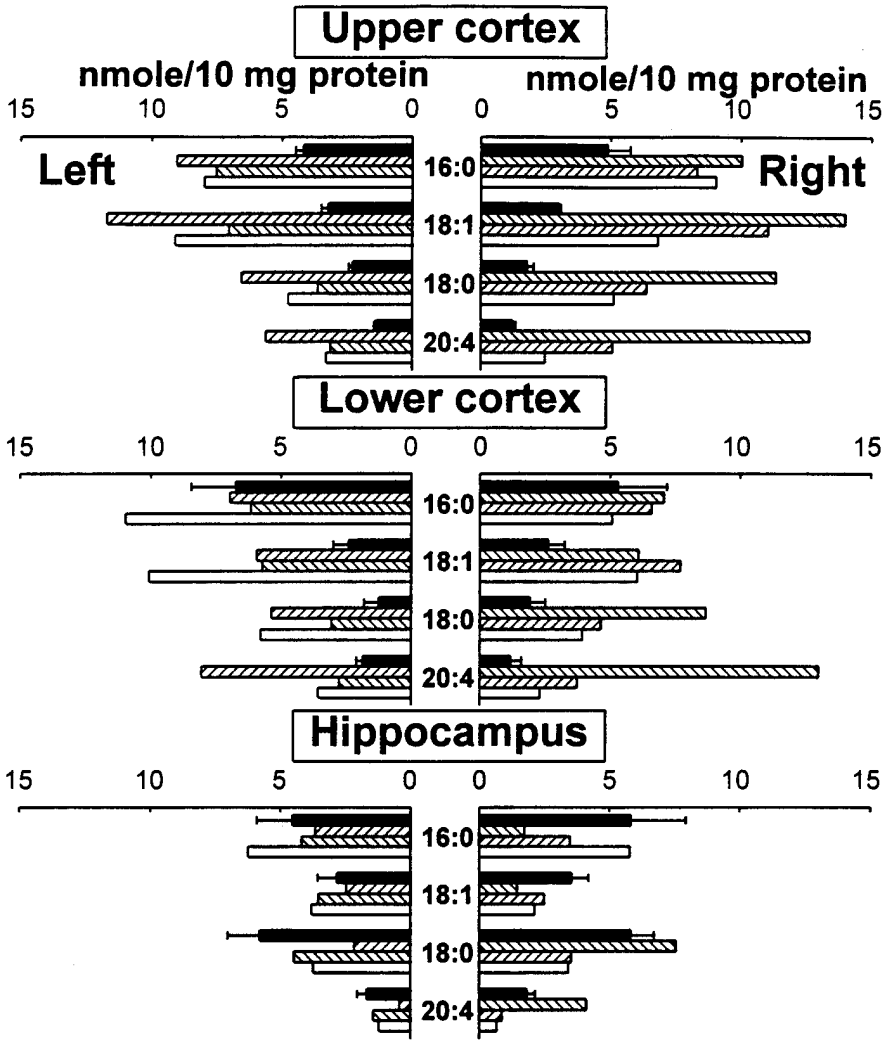
Black bars: Sham mean values ± SD (n=3)

Figure 31: Diacylglycerol acyl groups in the rat cerebral cortex and hippocampus at day 4 post-trauma



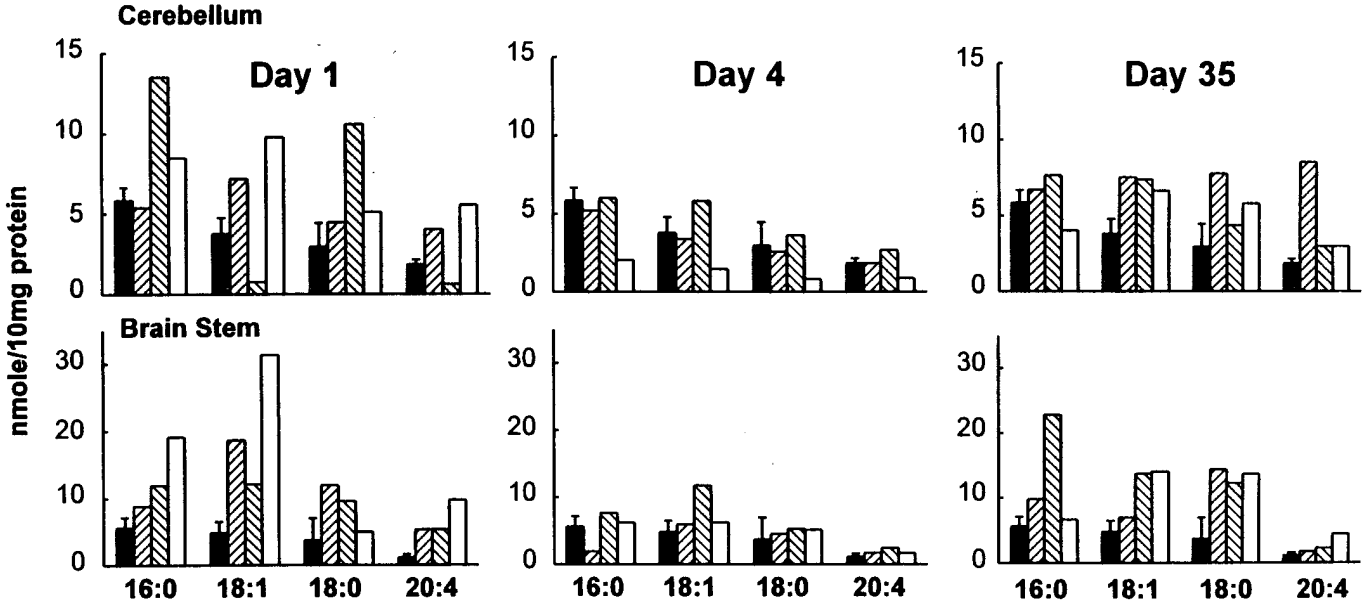
Black bars: Sham mean values ± SD (n=3). Other details as in Figure 29A legend.

Figure 32: Diacylglycerol acyl groups in the rat cerebral cortex and hippocampus at day 35 post-trauma



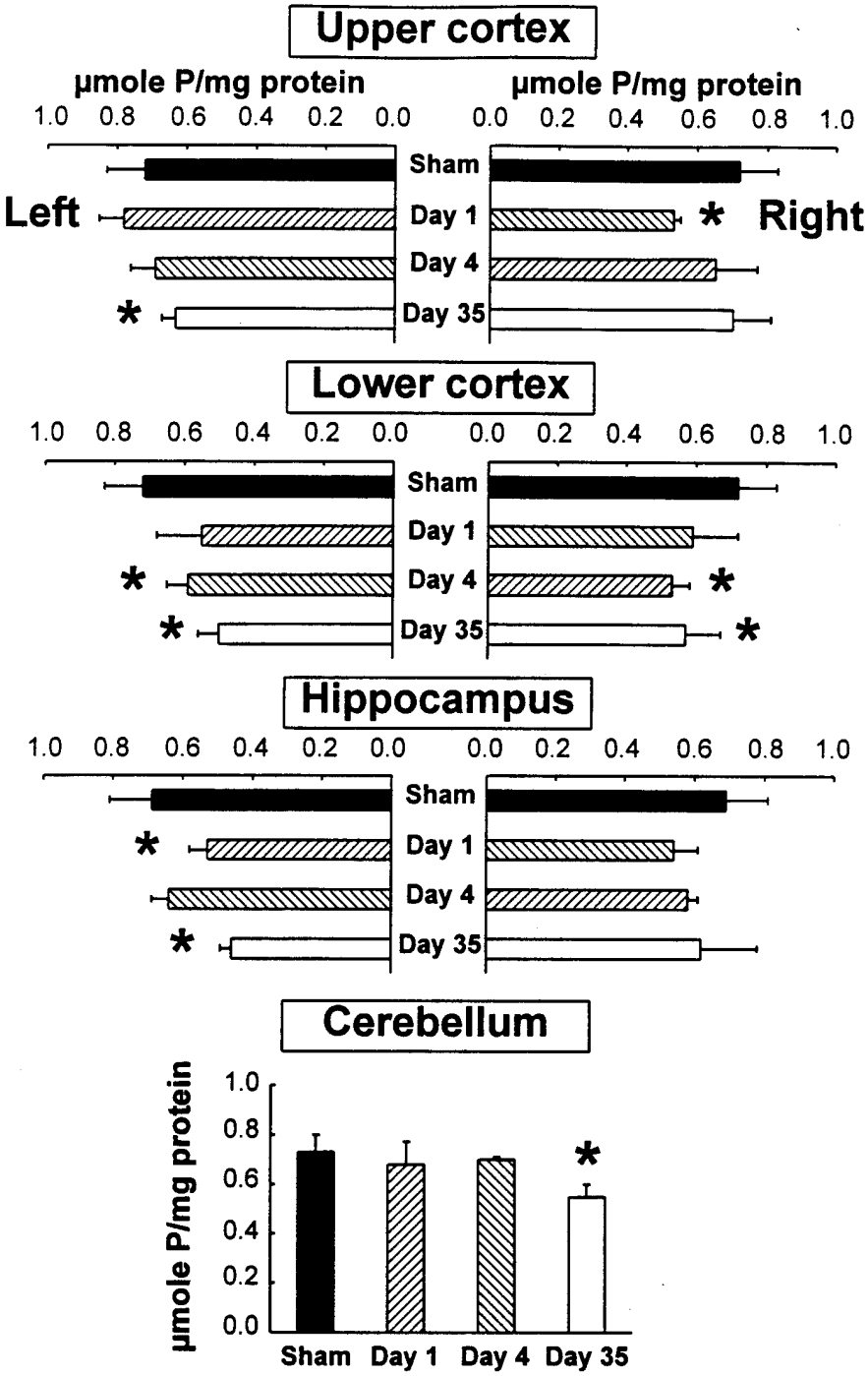
Black bars: Sham mean values ± SD (n=3)

Figure 33: Diacylglycerol acyl groups in the rat cerebellum and brain stem of rats after traumatic injury



Black bars: Sham mean values ± SD (n=3)

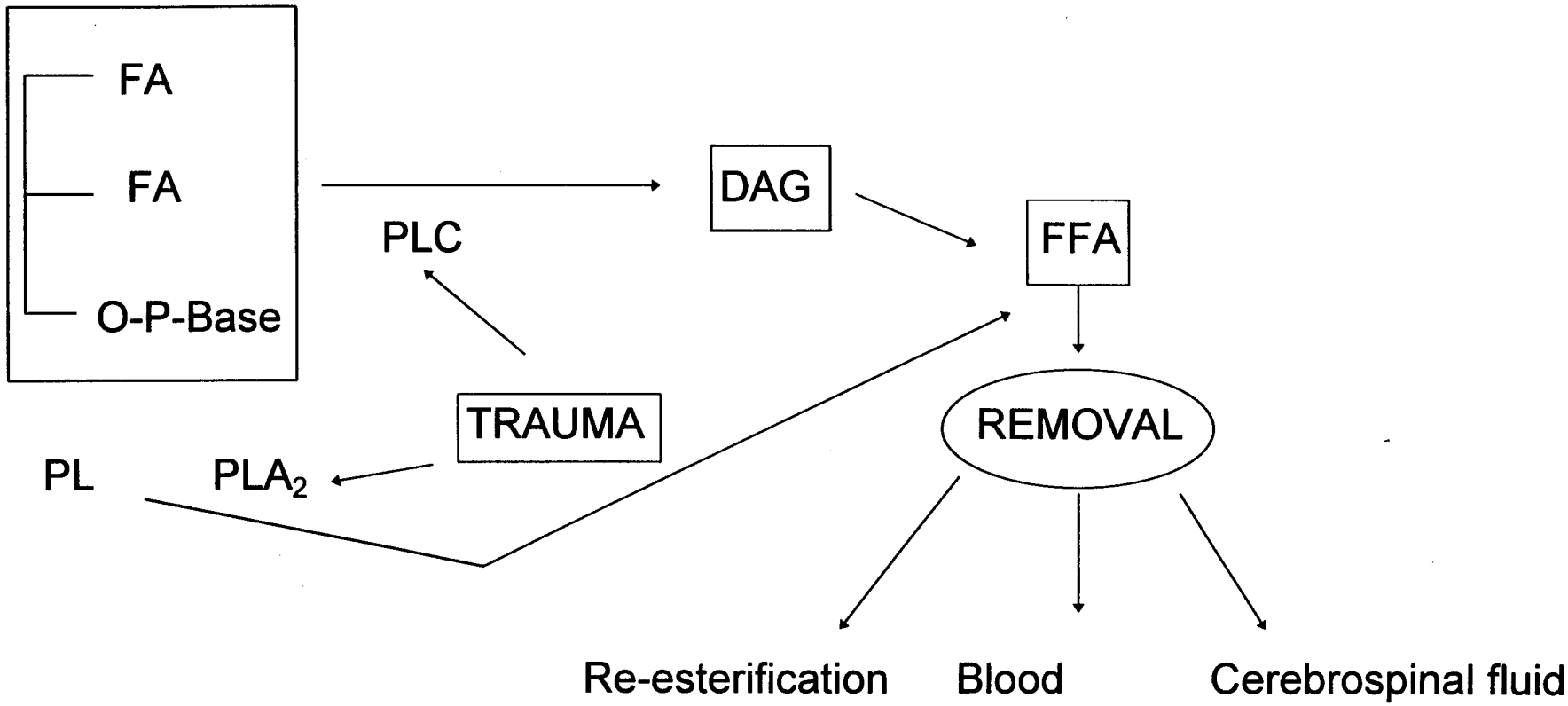
Figure 34: Changes in total lipid phosphorus content in brain areas after traumatic injury



Bars represent mean values ± SD from the following number (n) of individual determinations. Sham cortex, n=20; Sham hippocampus, n=10; Sham cerebellum, n=5; experimental animals at day 1, 4, and 35 post-trauma, n=3. Asterisks denote statistically significant differences from Sham values ($p<0.05$ Student's *t* test).

Figure 35

Traumatic injury activates phospholipids degradative pathways in neuronal cells



Abbreviations: FA, fatty acid; DAG, diacylglycerol; FFA, free fatty acids; PL, phospholipid; PLC, phospholipase C; PLA_2, phospholipase A_2; Base, i.e. choline (in phosphatidylcholine), inositol (for phosphatidylinositol), etc.

Discussion

The present study demonstrates that a consequence of right cerebral sensorimotor impact injury (right upper cortex), lipid metabolism is greatly affected not only in the cortical tissue surrounding the injury but also in contralateral and lower cortex, cerebellum, and brain stem. The accumaulation of PUFA 30 min after the injury in the right hemisphere may have been caused by the activation of PLA_2. An increase in PLA_2 activity in rat cerebral cortex has been reported 15 min after injury induced by a weigh drop device (Shohami et al., 1989). The profile of FFA and DAG accumulation after trauma varied among different brain areas. FFA peaked at one day in the right lower cortex, both hippocampi and brain stem 1 day after injury. FFA remained elevated in right and left upper cortices and contralateral hippocampus 4 days after injury.

Accumulation of DAG in cortical areas mainly ipsilateral with the injury, plus cerebellum and brain stem, was evident at day 1 with a transitory lowered basal values by day 4 and subsequent increase by day 35. **These long lasting changes in FFA and DAG levels may be the result of sustained activation of neuronal degradative pathways.**

Activation of degradative enzymes i.e. phospholipase A_2/A_1 (PLC A_2/A_1) and phosphilipase C (PLC) triggered by the impact will result in degradation of membrane phospholipids and accumulation of FFA and DAG [155]. The latter can be further degraded through a sequential DAG-lipase and monoacylglycerol lipase) to FFA and glycerol, figure 35.

Based upon the observed increase in FFA and DAG, however, we may underestimate the magnitude of phospholipid hydrolysis, since FFA are actively removed from the brain through the blood and the cerebrospinal fluid [156]. These processes must be very active, especially when massive lipid hydrolysis occurs during neuronal degeneration and death. As an example, on the first post injury day, the right upper cortex surrounding the injury showed a 26% decrease in phospholipid (PL) content (measured as micromoles of lipid phosphorus per mg of protein, Fig. 34). A 26% decrease in PL by day 1 accounts for 185 n moles P/mg protein and 370 n moles fatty acids/mg protein (considering that for each mole of PL there is one mole of phosphorus and 2 moles of fatty acids). The total n moles of FA accumulated as FFA plus DAG at day 1 post-trauma ranged from 1 to 3 n mole/mg protein. Therefore less than 1% of total acyl group lost from membrane PL were recovered in the brain samples as FFA and DAG products. This may also explain the variability among animals in the post-traumatic levels of FFA and DAG.

Changes in total PL/mg protein varied between areas of the brain but displayed less variability between individual experimental animals (Fig 34). In the right upper cortex (surrouding the injured area) the decrease observed by day 1 was not apparent by day 4 and 35. This may reflect an early massive degradation of PL (day 1) followed by necrotic cell death (i.e. loss of cell proteins and lipids). The remaining cortical cells will maintain similar phosphorus/protein ratio. **The changes observed at later times after trauma in other brain areas and the magnitude of these changes suggest a progression of the lesion with the time after injury from right upper cortex to left lower cortex and hippocampus to right lower cortex and cerebellum.** Histological analysis of different brain areas included in the present report support this profile of progressive degradative processes (see cell counts, section 2C, axonal degeneration section 2D of this report). Changes observed at later times (35 days) may reflect delayed cell death (apoptosis) in areas far from the injured cortex. The mechanisms which contribute to the spreading of the message and subsequent cellular death are not yet clear. However, the "window of time" between the early necrotic cell death in the area of the impact and the delayed, more generalized cellular degeneration and death, will allow us to explore new potential drugs to minimize long-term neurological deficits triggered by of the impact brain injury.

Synaptic phospholipids are highly enriched in long chain polyunsaturated fatty acids (PUFA) (i.e. 20:4n-6 and 22.6n-3) which are derived from dietary precursors, 18:2n-6 and 18:3n-3 [157]. The liver is actively involved in the synthesis of 22:6n-3 (docosahexaenoic acid, DHA) and its delivery to central nervous system (CNS) [158,159]. **The loss of PUFA from membrane PL, as a consequence of traumatic injury opens the possibility of post-traumatic dietary treatment with PUFA especailly n-3 fatty acids that are normally present at low levels in diet as compared to n-6 fatty acids [160].** This will favor the delivery 22:6n-3 and also 20:4-n-6 to the CNS and their ultilization for the resynthesis of neuronal PL essential for normal functional recovery of the brain.

III. SPECIFIC METHOD OF PRODUCING BRAIN STEM DYSFUNCTION CAUSING APNEA

Background

Apnea often follows severe brain injury and may be the cause of death following a brain injury not in itself a fatal one [56,161-163] We have attempted to develop a post traumatic apnea paradigm in our rat injury model in order to treat post traumatic apnea in humans. An apneic response varying with piston impact intensity is not easily produced in the rat. It may take up to 450 gms dropping one meter onto a rat's head to produce this effect! [164] Thus far we have tested lateral skull and anterior and posterior convexity piston impact sites in an attempt to produce a graded response to traumatic brain injury. Lateral impacts caused petrous bone disruption and direct damage to the brain stem by skull base fragments. We have attempted 2 types of vertex impacts to produce apnea: 1) through the same right sided craniectomy we utilize in our experiments to produce cortical damage and 2) by a more posterior vertex blow using a 6 mm circular impactor striking the intact skull just ahead of the lambdoid suture. In all instances the animal is anesthetized with urethane (1.5 mg/kg) because urethane is not a respiratory depressant. The animals are fixed in the stereotaxic frame and impact severity is controlled by varying the depth of piston excursion from 1 mm to 4 mm. After impact strike apnea onset and duration are recorded.

Results of Efforts to Produce Traumatic Apnea

Table 4 shows the length of apnea produced by various impactor depths delivered either anteriorly through the standard craniectomy onto the exposed dura or posteriorly onto the intact skull.

TABLE 4

Posterior Impact (Skull Intact)			Anterior Impact (Through Craniectomy)		
#	Impact Depth	Length of Apnea	#	Impact Depth	Length of Apnea
339	4 mm	4′ 29"	450	4 mm	2′0"
340	4 mm	4′ 05"	451	4 mm	25′0"
347	4 mm	4′ 30"	452	4 mm	5′0"
453	3.5 mm		453	3.5 mm	0′ 0"
454	3.5 mm		454	3.5 mm	0′ 8"
455	3.5 mm		455	3.5 mm	0′ 8"
456	3.5 mm		456	3.5 mm	0′ 4"
341	3 mm	8′ 11"	449	3 mm	0′ 5"
342	3 mm	11′ 38"	457	3 mm	0′ 5"
348	3 mm	22′ 0"	458	3 mm	0′ 5"
344	2 mm	1′ 31"			
345	2 mm	0′ 36"			
346	1 mm	2′ 10"			

Because a graded apneic response corresponding to differing impact depths has been hard to achieve, we have ceased attempting to obtain this effect. Rather, we have begun electron microscope studies of the periaquaductal area and floor of the 4th ventricle following a 4 mm deep anterior impact through a craniectomy. This is the injury site for virtually all of our other experiments. This injury produces significant apnea, table 4.

After we determined that a 4 mm deep cortical impact through a craniectomy would produce apnea from 2 to 25 minutes, separate rats were anesthetized, prepared, and impacted at 4mm. Within minutes of impact the animal was perfused with 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer set at pH 7.35. The brain was allowed to fix in the head for one week prior to removal. The brain stem was exposed by carefully trimming away the cerebellum, slicing through the cerebellar peduncles with a double- edged razor blade to minimize torsion on the brain stem. Following a wash in six changes of 0.1 M phosphate buffer with 8% sucrose and three

changes of 0.1M cacodylate buffer with 8% sucrose, the brain stem was divided into two pieces by slicing through the level of the superior colliculi to expose the cerebral aqueduct in the first piece. The floor of the IV ventricle was incorporated in the second piece. All remaining connective tissues were removed to expose the surface of the IV ventricle. These blocks of brain stem were then osmicated for two hours, washed overnight in cacodylate buffer, dehydrated in graded ethanols, and critically point dired in a Tousimis Samdri 790 dryer. Each block was then glued to a stub and coated with gold-palladium in an Anatech sputter coater. The blocks were then examined and photographed in a Jeol T-300 scanning electron microscope (SEM).

Results

Scanning electron microscope views of the aqueduct and fourth ventricular floor of control animals revealed an intact ependymal lining on all ventricular surfaces characterized by fields of cilial projections and minimal amount of particulate matter. Following piston impact on the right sensorimotor cortex which depressed the brain 4 mms.

Following sensorimotor cortical injury known to cause apnea the aqueduct and floor of the fourth ventricle were seen to have sustained major injury, figures 37 to 43, pages 94-99.

Discussion

These SEM pictures support Duret's > 100 year old hypothesis that following traumatic brain injury a fluid wave jets through the aquaduct and impinges with great force on the sides of the aquaduct and on the floor of the fourth ventricle [165,166]. The cracks and fissures on the floor of the fourth ventricle may well extend downward into the respiratory nuclei some of which lie just under the floor of the fourth ventricle. Such tears may well effect the respiratory nuclei and account for apnea which so often accompanies head injury.

Further studies will be necessary to further evaluate these changes.

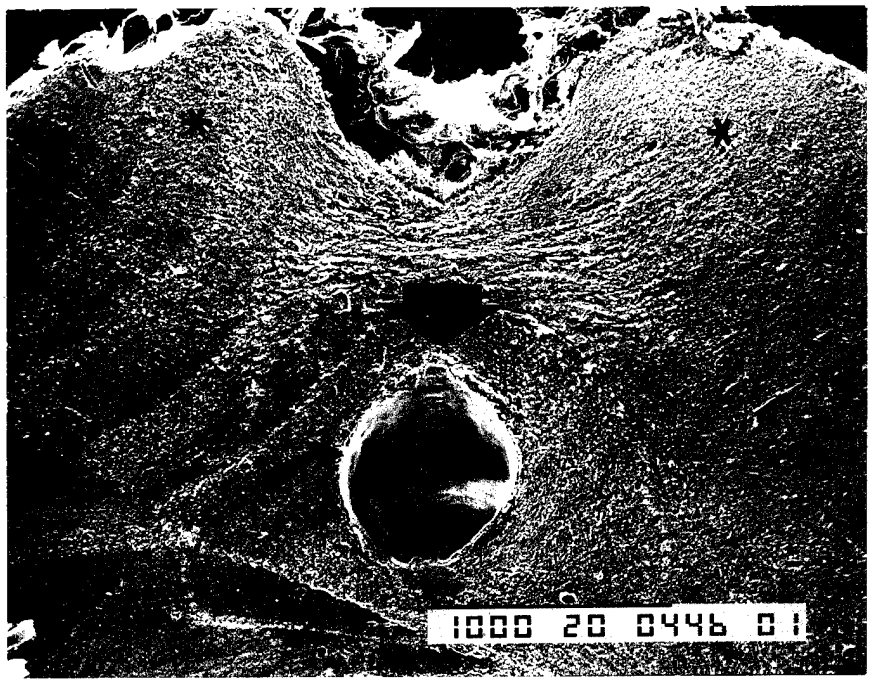


Figure 36: A slice through the midbrain at the level of the superior colliculi (asterisks) reveals the interior of the cerebral aqueduct (large arrow). The small arrow points to the ependymal lining of the aqueduct. Bar = 1 mm.

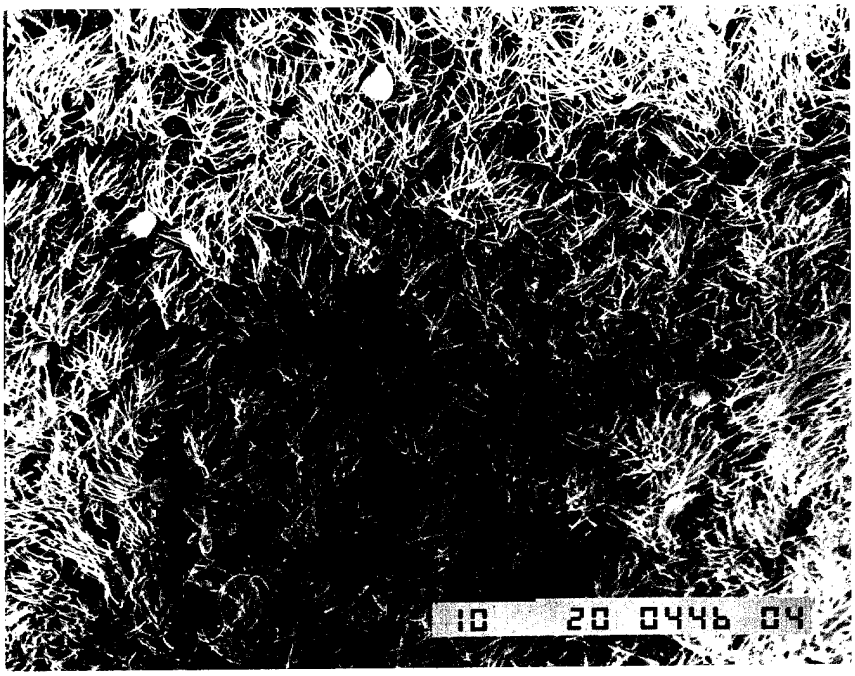


Figure 37: Cilia extend from the apices of the ependymal cells into the aqueduct providing an almost uniform lining of this surface. No fissures are evident. Bar = 10 um.

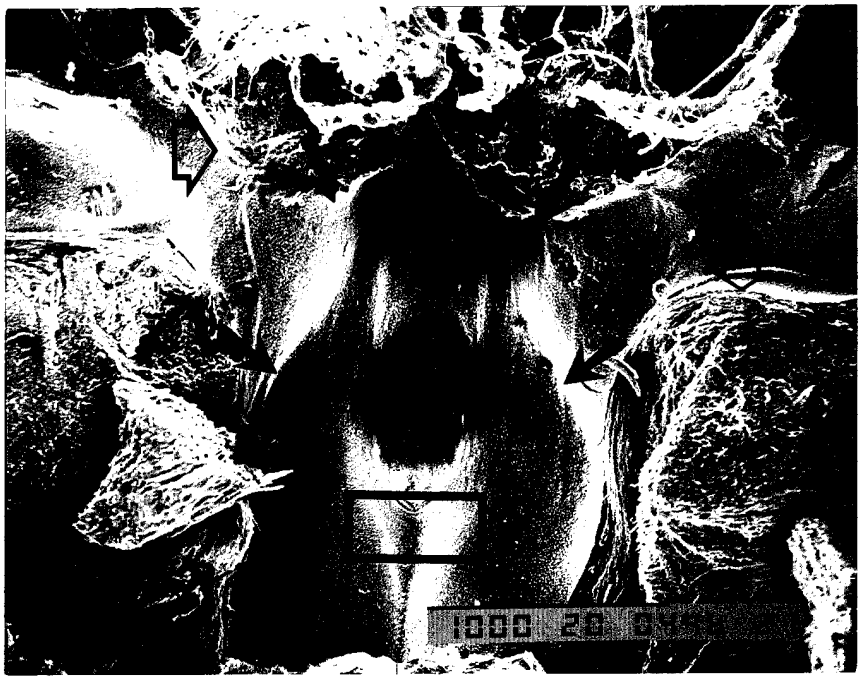


Figure 38: Brain stem from a normal control animal with cerebellum removed to expose the IV ventricle delineated by solid arrows. The large open arrow points to the anterior medullary velum and the small open arrow indicates a middle cerebellar peduncle. The rectangle indicates the region shown at higher magnification in the next figure. Bar = 1 mm.

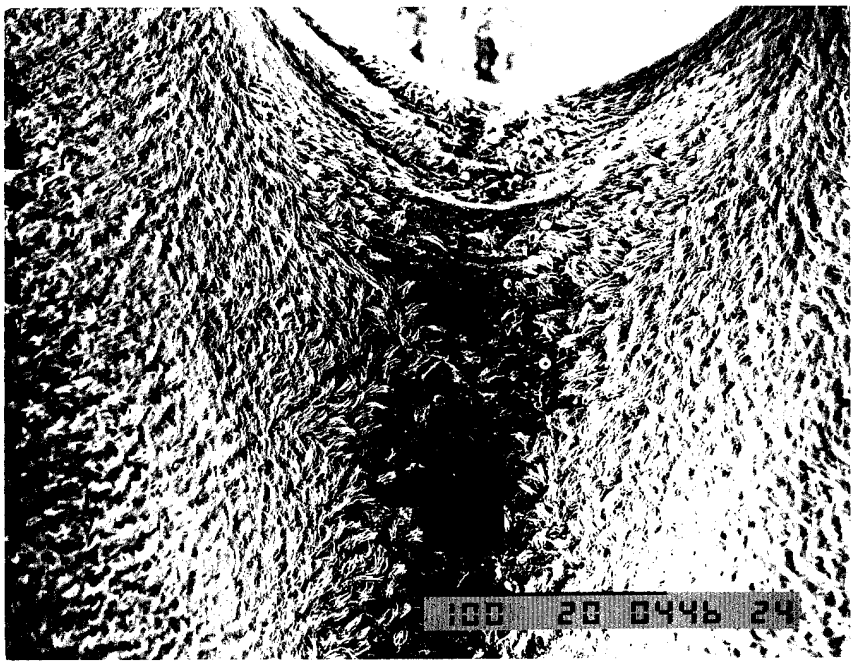


Figure 39: The abundance of cilia in the floor of the IV ventricle is evident. The large solid arrow indicates the midline sulcus the surface of which is devoid of these projections.

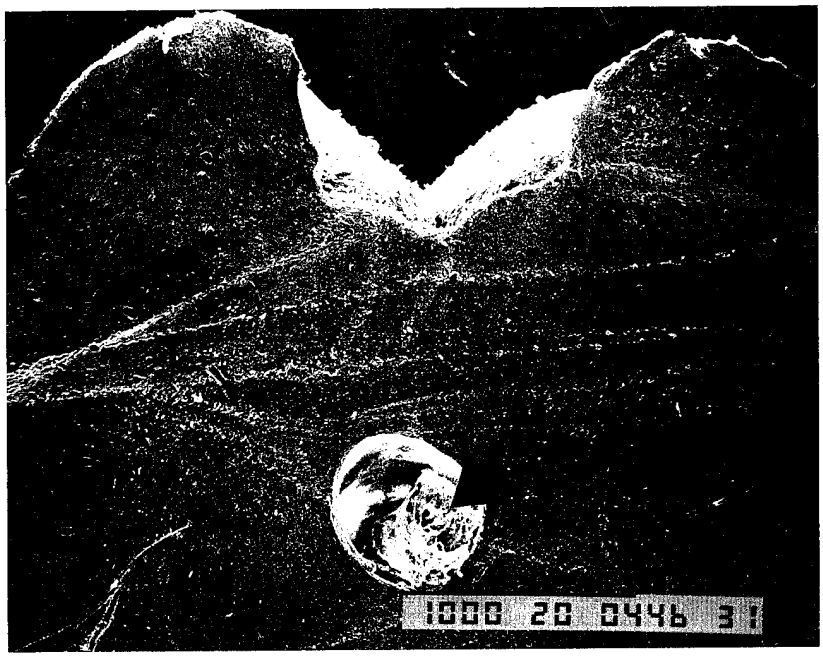


Figure 40: The cerebral aqueduct is shown in a preparation of a midbrain taken from a rat subjected to impact which depressed dura and cortex 4 mm. A large fibrin clot lies within the aqueduct. This clot does not totally occlude the aqueduct. Bar = 1 mm.

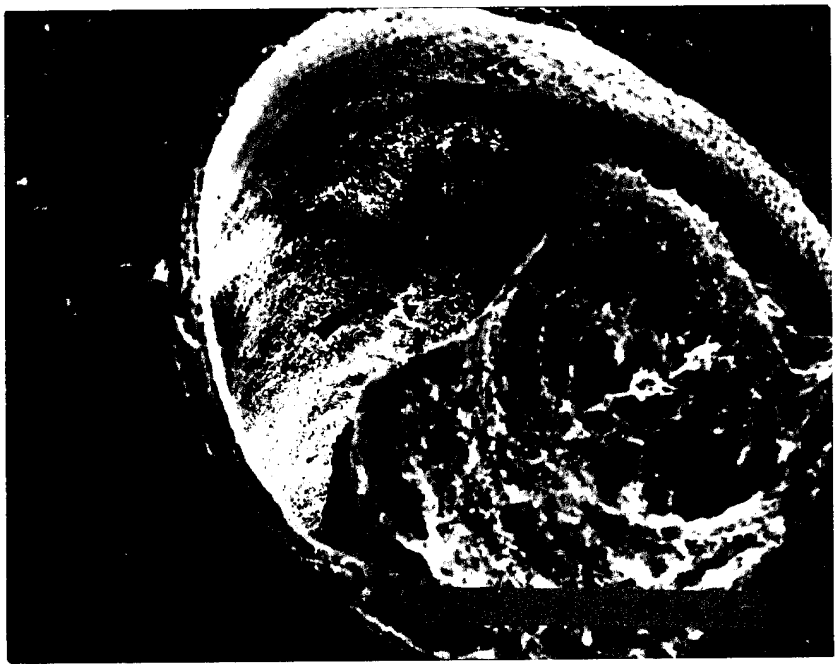


Figure 41: At higher magnification, a fissure (arrow) appears on the wall of the aqueduct. Bar = 100 um.

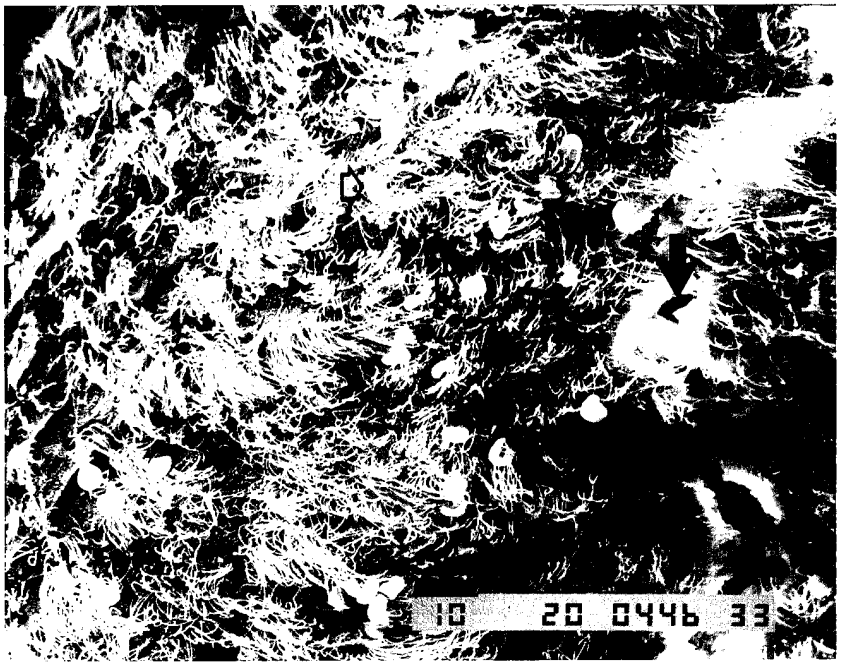


Figure 42: At even higher magnification, the fissure is more apparent. It penetrates deeply into the lining of the aqueduct (arrow). Scattered red blood cells (small open arrows) are attached to the cilia along the canal. Bar = 10 um.

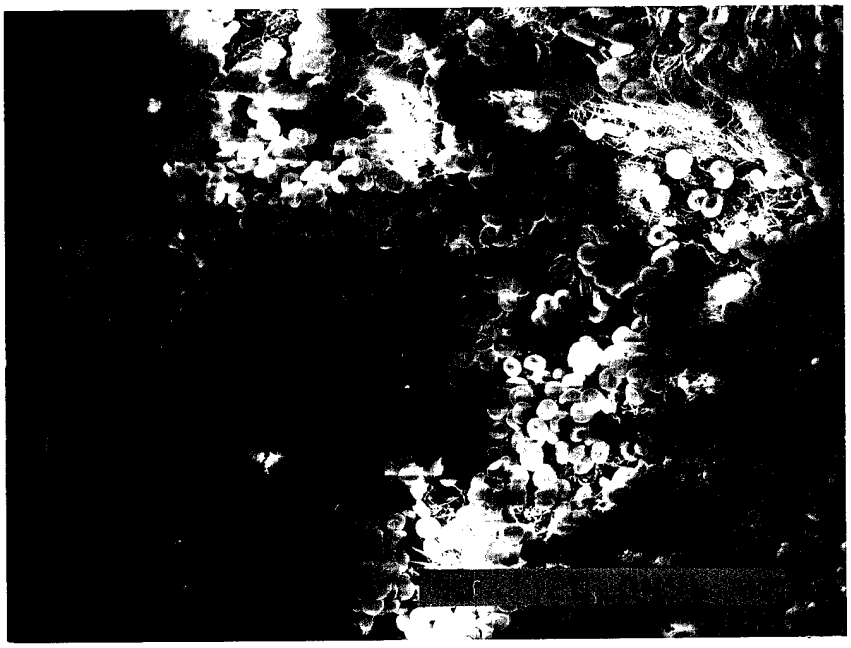


Figure 43: The floor of the IV ventricle of an animal subjected to a 4 mm impact reveals extensive evidence of trauma including a fissure (arrow) and concentrations of red blood cells. Bar = 10 um.

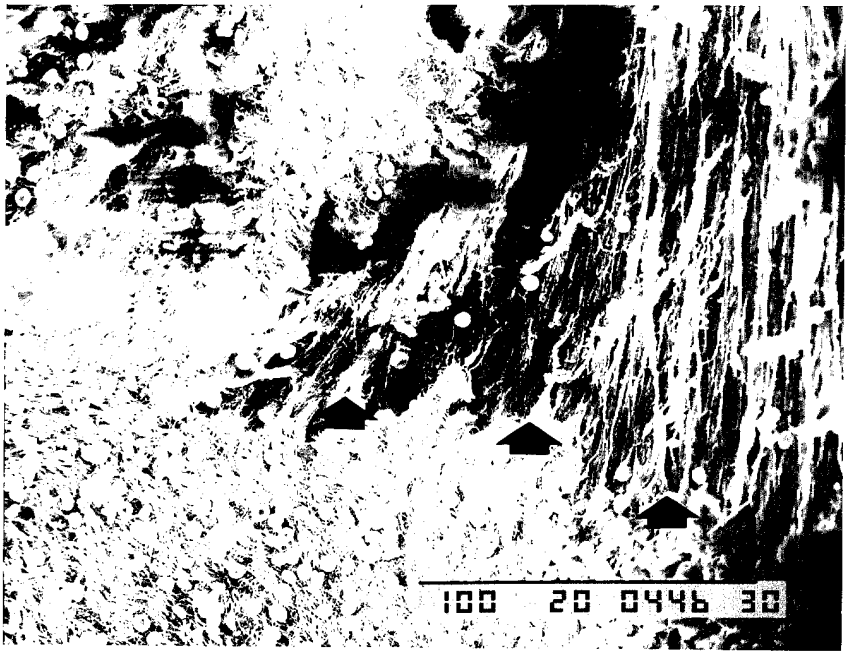


Figure 44: In other regions, the ependymal cells and cilia have been sheared away entirely (delineated by arrows) leaving only the presumptive astrocytic lining and strands of amorphous material which may repesent fibrin. Bar = 100 um.

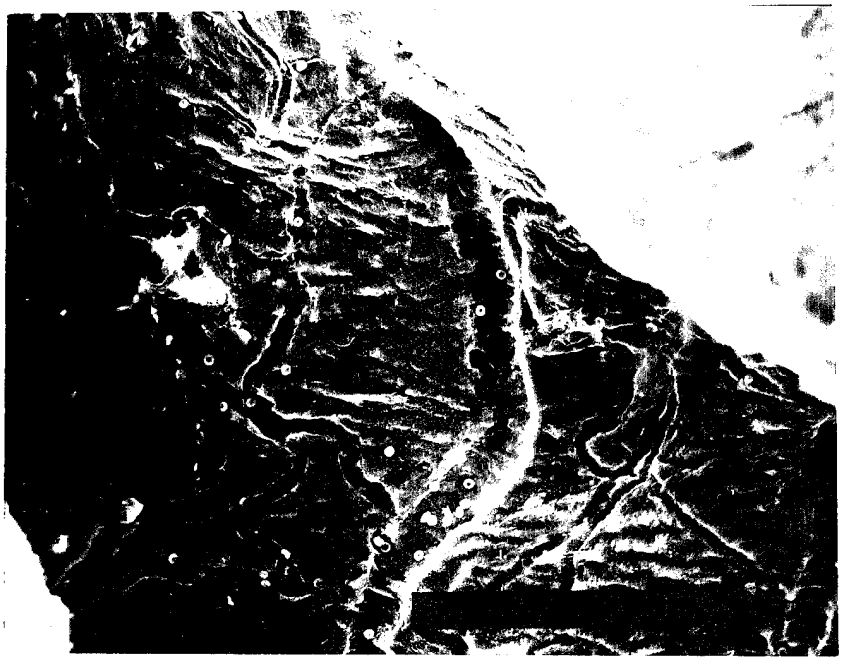


Figure 45: Some regions show extensive loss of the ependyma with only an impression of the subependymal vessels remaining. Bar = 100 um.

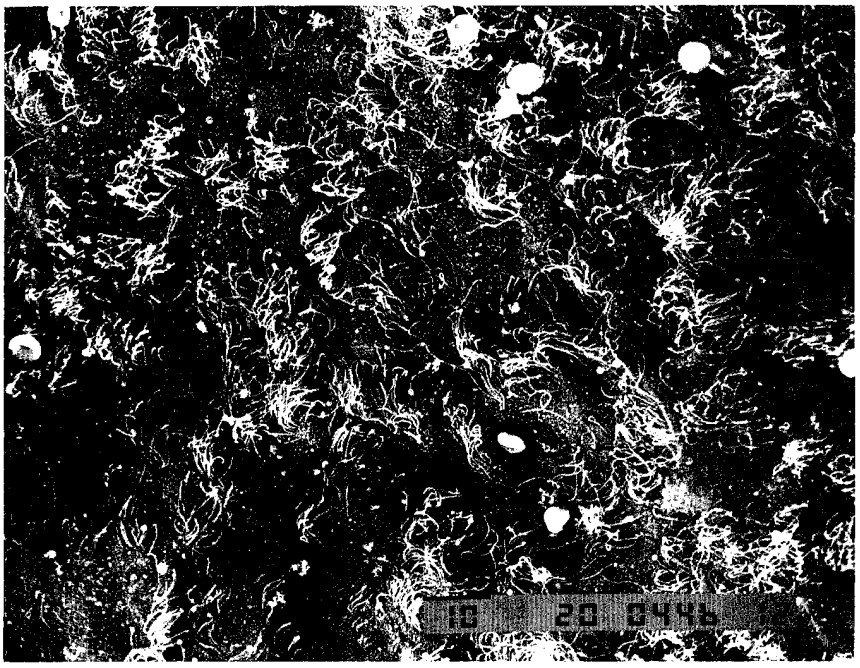


Figure 46: In other regions of the IV ventricle, the ependymal lining remains but large numbers of the cilia are missing. Bar = 10 um.

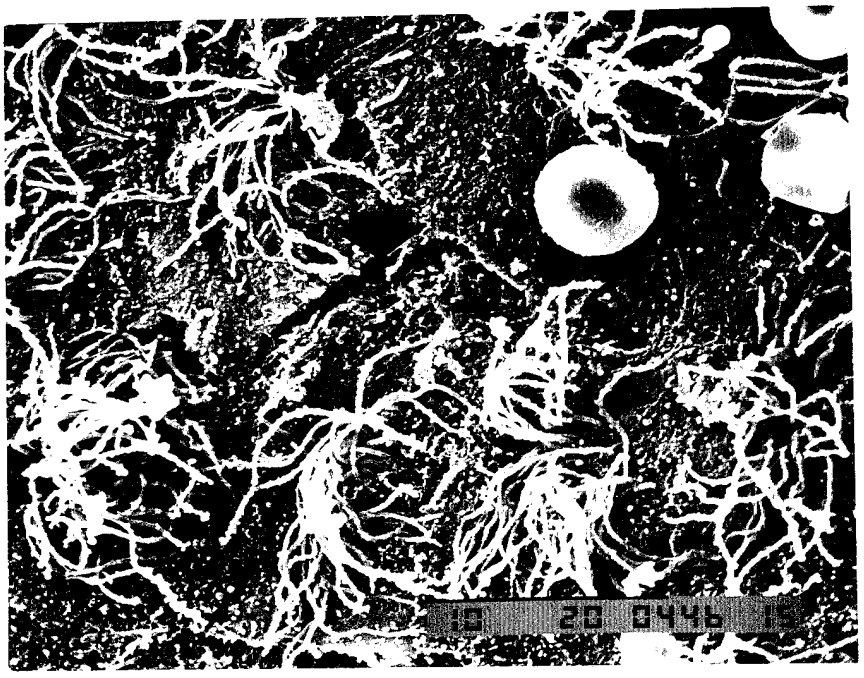


Figure 47: The floor of the fourth ventricle shows small cracks (solid arrow) and other imperfections, fragmented cilia (small open arrows) and red blood cells. Bar= 10 um.

CONCLUSIONS

1. We have successfully modified a model of piston-induced traumatic brain injury in anesthesized rats which provides a uniform, sensorimotor cortical injury from animal to animal.

2. Injured animals exhibit a contralateral hemiparesis lasting up to 35 days plus memory and spatial localization deficits for 17 days after injury.

3a. Histologic studies naturally show brain damage at the cortical impact site with loss of cortical mantle and gliosis. Additionally, however, cresyl violet-luxol fast blue stains strongly suggest wide spread cellular damage and cell loss throughout the entire cerebral cortex bilaterally but not in either hippocampus. Mild ventricular dilatation contralateral to the side of injury supports the possibility of wide spread cortical cell loss.

3b. Within minutes of impact injury the proto oncogene c-Fos is widely activated throughout the cerebral cortex and ipsilateral hippocampus probably via excitatory amino acids released by the trauma acting upon NMDA receptors.

3c. Silver stains reveal that after sensorimotor cortical injury widespread degeneration occurs within the entire subcortical motor system including the cerebellum. We feel that these findings can only be explained by **transsynaptic degeneration** which we are unaware as having been described before for the motor system.

4. Free fatty acid and diaglyceride increase acutely about the injury site while total lipid phosphorous decreases. This indicates neural degradation in brain about the impact area. These changes also occur widely throughout the brain including the contralateral basiventral cortex, contralateral hippocampus, brain stem and cerebellum. These changes, indicating neural, degredation occur in various brain areas including the cerebellum up to 35 days after injury.

5. Scanning electron micrographs of the aquaduct and floor of the fourth ventricle after severe cortical impact sufficient to cause apnea reveal widespread shearing and denuding of ependymal cilia plus fissuring of the aquaductal wall and floor cells and of the fourth ventricle.

SIGNIFICANCE OF FINDINGS

1. The occurrence of both widespread cortical cell loss and widespread transsynaptic degeneration within the entire subcortical motor system have profound implications for how the brain must reorganize to achieve any recovery after traumatic injury. Compensation (by plasticity or whatever other means) must occur not only at the cortical level but also within various subcortical projection systems if the animal is to show recovery after injury. **It may be hypothesized that an optimal neuroprotective drug must not only aid damaged cells about the site of injury but also aid affected cells throughout the entire cerebral cortex and protect subcortical functional systems from transsynaptic degeneration.**

2. Improvement of sensorimotor function after injury shows an initial fast return for 5 to 7 days followed by a slower rate of return lasting many weeks thereafter. The differing slopes of the "initial" and "later" improvement phases suggest that different biologic mechanisms may underlie "initial" and "later" behavioral improvement. For instance, "initial" improvement may occur as damaged but not destroyed brain gets over the early effects of brain injury (e.g. spreading depression, effects of excitatory biogenic amines, vasogenic brain edema). "Later" improvement may reflect brain reorganization or neural recovery.

   Different neuroprotective or neuroenhancing drug strategies may be required for the "initial" and "later" improvement phases.

   Improvement after brain injury may reflect either true neural recovery or an animal's adaptation to neural damage. Neuroprotective drugs could aid true neural recovery or foster more efficient behavioral adaptation.

3. Free fatty acid, diaglyceride, and lipid phosphorous data indicate that after focal traumatic brain injury widespread neural degeneration occurs not only about the impact site but in other distant brain areas as well. This loss continues for at least 5 weeks after focal brain injury. These biochemical findings strongly support our histological data showing widespread cortical cell loss and transsynaptic degeneration 8 weeks after injury which extends into

the cerebellum. Since cell death appears to occur in brain areas quite distant from the injury site many weeks following injury, possibly neuroprotective drugs should be given for weeks after injury in an attempt to decrease delayed cell death. **If these findings are born out they would fundamentally change the timing of neuroprotective drug administration to help brain injured people.** Currently neuroprotective drugs are given only within hours of injury and not over an extended time period.

4. Scanning electron microscope images show widespread shearing of fourth ventricular ependymal cells and their cilia after severe sensorimotor cortical impact. These findings suggest that with cortical impact injury a hydraulic jet of ventricular fluid exists the aqueduct. This jet may literally shear off the fourth ventricular ependymal cells or their ciliated processes. These findings tend to support the 150 year old theory of Duret that head injury causes such a CSF effect upon the fourth ventricular floor and medulla.

The widespread cracking, fisuring and tearing of the fourth ventricular floor following traumatic injury may explain post traumatic apnea because some components of the medullary respiratory complex lie just under the floor of this ventricle. If these subependymal respiratory structures are torn and shorn apart, neural circuits necessary for respiratory cycle maybe rendered inoperative. **Actual mechanical disruption of respiratory neural circuits might be the anatomic correlate of irreversible apnea.**

FUTURE RESEARCH DIRECTIONS

We feel that we should continue the basic thrust of our brain injury experiments as outlined in our projected work statement, Appendix 2. We feel that our multidiscipline approach provides the optimal milieu for the study of traumatic brain injury. Our research groups are synergistic and provide each other mutually supportive insights. For instance, histological studies strongly suggest continued cell damage for many weeks after injury and show definite fiber track degenerating at 8 weeks post injury. These findings make the neurochemical studies of Dr. Bazan's group (which showed evidence of protracted neuronal death for 35 days after injury) much more understandable. Behavioral recovery after injury occurred despite a devastated sensorimotor cortex. Whatever mechanisms of recovery are taking place subcortical structures are probably involved and should be examined histologically and neurochemically. The histological evidence of transsynaptic degeneration provides a firm basis for brain sampling for the investigation of biogenic amines and catecholamines. Electron micrographs have provided strong reasons for the occurrence of apnea after traumatic brain injury.

Ideally in the future when drugs which might ameliorate the effects of traumatic brain injury are evaluated histological and neurochemical correlated of their efficacy will be obtained. This would be the sought for ideal.

Since our histological data strongly suggest widespread cell death in many cortical regions consequent to focal brain injury and axon degeneration studies indicate transsynaptic degeneration which may also be associated with cell death, our future studies, histologically speaking, will concentrate on counting cortical and subcortical cell populations (eg in striatum or cerebellum) to look for actual cell number decreases consequent to trauma.

Neuroprotective drugs should preserve cell populations and preservation of at risk cells by drugs purported to be neuroprotective would be a powerful way to evaluate neuroprotective drugs <u>in</u> <u>vivo</u>.

We will decide what neuroprotective drugs to test (both for cortical injury and to protect against apnea) and how they will be given at a later date.

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APPENDIX A

TABLE 1

Score	Neurologic Scoring System For Beam Traversing
1	Unable to traverse or place BOTH the affected hindpaw and forepaw on the horizontal surface of the beam
2	Unable to traverse but places EITHER the affected hindpaw or forepaw on the horizontal surface of the beam and can maintain balance for at least 5 seconds
3	Unable to traverse but places BOTH the affected hindpaw and forepaw on the horizontal surface of the beam and can maintain balance for at least 5 seconds
4	Traverses beam but DOES NOT PLACE EITHER of the affected paws on the horizontal surface of the beam
5	Traverses the beam and AT LEAST ONCE PLACES EITHER the affected hind OR forepaw on the horizontal surface of the beam (i.e. Each affected limb is on the horizontal surface for less than half the steps)
6	Traverses the beam but can place ONLY ONE AFFECTED PAW on the horizontal surface of the beam (i.e. only one paw is on the horizontal surface for more than half the steps while the other is on the horizontal surface for less than half the steps, grasps the vertical surface or is dragging)
7	Traverses the beam with BOTH affected paws on the horizontal surface of the beam for more than half its steps
8	Traverses the beam with BOTH affected paws on the horizontal surface of the beam, neither paw ever graps the vertical surface and there are no more than two footslips. Toe placement style is the same as pre-injury

APPENDIX B

FIVE-YEAR SOW STATEMENT

YR 01:

1. Develop and characterize rat head injury model at different injury energy levels.

* a) Physiological effects of brain trauma: MABP, Heart Rate, End-Tidal CO_2, Blood Gases, Respiratory Rate (n=64).

* b) Variance of physiological response to brain trauma as a function of anesthetic: Halothane vs Urethane (n=60).

O c) Histological evaluation of head trauma: Light Microscopy, C-fos Analysis and Electron Microscopy of respiratory nuclei including the surface of the fourth ventricle) (n=40).

YR 02:

1. Evaluation of the acute effects of brain trauma, including apneic responses, at different injury energy levels: Neurotransmitters and Histology.

a) Regional effects of acute trauma on excitatory amino acids in the cortex and brain stem (n=32).

b) Regional effects of acute trauma on catecholamines and indoleamines in the cortex and brain stem (n=32).

O c) Histological evaluation of head trauma: Light Microscopy, C-fos Analysis and Electron Microscopy of respiratory nuclei including the surface of the fourth ventricle) (n=40).

O 2. Evaluation of the acute effects of head injury on: a. synaptic phospholipase A_2 b. inositol lipids particularly diacylglycerol IP_3 and c. turnover in membrane phospholipids by <u>in vivo</u> labeling of arachidonoyl-phospholipids (n=100).

YR 03:

1. Cortical and brain stem neurotransmitter effects of brain trauma at different energy levels up to two hours after the initial impact injury. Cortical measurements will be useful in correlating neurotransmitter results with future behavioral recovery experiments. Brain stem measurements will be correlated with the resumption of breathing in artificially ventilated animals.

a) Evaluation of excitatory amino acid response in the cortex by in-vivo microdialysis (n=60).

b) Evaluation of excitatory amino acid response in the brain stem by in-vivo microdialysis (n=60).

* = COMPLETED; O = ONGOING

YR 03 cont'd:

c) Evaluation of the catecholamine response in the cortex by by in-vivo microdialysis (n=60).

d) Evaluation of the catecholamine response in the brain stem by in-vivo microdialysis (n=60).

e) Effects of drugs which may enhance recovery of spontaneous breathing in head injured-artificially ventilated rats (n=72)

2. Evaluation of the acute effects of head injury on degradative enzyme activation by assessing endogenous changes in pool size and composition of free polyunsaturated fatty acids and diacylglycerol. The time course of platelet activating factor (PAF) accumulation will also be determined by HPLC analysis (n=100).

YR 04:

1. Long term effects of brain trauma on neurological/behavioral parameters and neurotransmitters in recovering animals. Recovering animals will be evaluated at 6 hr, 1, 2, 4, 7, 10, and 14 days after brain injury. Correlations will be made between regional neurotransmitter alterations and degree of behavioral/neurological recovery.

   a) Neurological and behavioral characterization of low energy injury level (n=40)

   b) Evaluation of excitatory amino acids and catecholamines in recovering animals. Neurological and behavioral testing will also be conducted in the same animals (n=162).

2. The acute effects of head injury on the precursor of platelet activating factor (PAF) at the excitable membrane and its conversion into PAF will be evaluated by radiolabeling/HPLC-spectrometry assays (n=100).

YR 05:

1. Effects of drugs which may enhance neurological/behavioral recovery in head injured rats.

   a) Various drugs will be tested in head injured rodents. Animals will be evaluated for neurological/behavioral recovery followed by subsequent analysis of cortical and brain stem excitatory amino acids and catecholamines. Correlations will be made between the effects on excitatory amino acids or catecholamines and neurological/behavioral recovery (n=300).

2. The effects of platelet activating factor (PAF) antagonists on phospholipases and PAF after acute head injury will be studied. The neurochemical effects of PAF will be correlated with their effects on neurological/behavioral recovery (n=100).

APPENDIX C

Discussion of Performance According to Time Schedule

Cortical Injury:

Our model of impact injury to the right sensorimotor cortex is well established. The produced impact injury is quite constant among rats. We have characterized spontaneous recovery of the motor and memory system after this injury. c-Fos studies are completed. By quantifying damaged cells in various cortical area after impact injury we have presumptive evidence of wide spread, cell loss. This cell loss appears to be protracted occuring many weeks.

Because the technician who cut brains in the LSU Department of Anatomy has decided to retire, getting brains processed in this department (as was our original plan) has been impossible since January, 1995. We, therefore, have turned to an outside source for brain processing. (Neuroscience Associates, Knoxville, Tenn). This has been a blessing in disguise because the commercial processor has done silver stains on our specimens. (We were not originally intending to do such stains). **The silver stain technique has allowed us to ascertain transsynaptic degeneration throughout the entire subcortical motor system after cortical sensorimotor injury.** This is very surprising and to the best of our knowledge an unreported finding which deserves continued evaluation.

Dr. Bazan's group has measured brain free fatty acids, diacylglycerol, and phospholipids and has found long lasting and wide spread effects in these components of neural cell membranes. These neurochemical studies have only been ongoing for the past 9 months because personnel required for these assays arrived only in September, 1994.

Brain Stem Injury:

We have also made fundamental scanning electron microscope findings. This imaging technique has shown the floor of the fourth ventricle to be severely disrupted after cortical impact. This disruption could account for apnea which is commonly observed after head injury. These findings, too, deserve further clarification.

In addition to developing our reliable and reproducable brain injury model and developing a 4 test battery to evaluate motor function, Dr. Soblosky also added a memory test to our armamentarium for evaluating brain injury and possible neuroprotective drugs. Memory is, perhaps, more a measure of higher cortical function than are locomotor tests. We did not anticipate developing and utilizing memory tests when we wrote this contract. Developing this important testa allows us to measure more subtle brain function but has caused us to fall behind in measurements of regional amino acids and catecholamines in cortex and brain stem after injury. Delay in undertaking these studies has been providential because there are literally hundreds of brain areas that could be assessed for these substances after injury and we have pondered long and hard about which brain areas to sample. With the recent return of silver stained brain sections showing widespread transsynaptic degeneration in the motor system we now have strong reason to sample various subcortical nuclei of the motor system. Dr. Soblosky has perfected the HPLC methods to assay both catecholamines and amino acids. Our intended O2 year work on measuring these substances in various brain regions following brain injury will commence within a few weeks. These substances will be measured adjacent to the cortical injury and in subcortical motor nuceli.

In summary: 1) Our model is developed and is ready to evaluate possible neuroprotective drugs; 2) Our histological work is about 3/4 done. We should be able to conclude this work in the next 6 to 9 months. 3) Neurochemical studies from Dr. Bazan's laboratory are about on schedule. 4) We are approximately 6 months behind in studying brain amino acids and catecholamines after brain injury. We expect to be "on target" in this department by year's end.